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OWN-ROOTED TREES OF JAPANESE  
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**PRODUCTION AND FIELD EVALUATION  
OF OWN-ROOTED TREES OF  
JAPANESE PERSIMMON  
(*Diospyros kaki* Thunb.)**

**2000**

**TAKUYA TETSUMURA**

# Contents

|   |       |           |
|---|-------|-----------|
| <b>General Introduction</b>   | ----- | <b>1</b>  |
| <br><b>Chapter 1. Production of Own-rooted Trees by Micropropagation</b>    |       |           |
| <b>Section 1. Improvements in Rooting of Microcuttings</b>                  |       |           |
| (I) Improvements by Using Shoots Subcultured In Vitro with BA               | ----- | 3         |
| Introduction  |       |           |
| Materials and Methods   |       |           |
| Results   |       |           |
| Discussion  |       |           |
| Summary   |       |           |
| (II) Improvements by Using Shoots Differentiated from In Vitro Roots        | ---   | 7         |
| Introduction  |       |           |
| (a) <i>High-frequency Shoot Differentiation from In Vitro Roots</i>         | ----- | 8         |
| Materials and Methods   |       |           |
| Results and Discussion  |       |           |
| Summary   |       |           |
| (b) <i>Characteristics of Shoots Differentiated from In Vitro Roots</i>     | ----- | 13        |
| Materials and Methods   |       |           |
| Results   |       |           |
| Discussion  |       |           |
| Summary   |       |           |
| <b>Section 2. Improvements in Acclimatization of Micropropagules</b>        | ---   | <b>23</b> |
| Introduction  |       |           |
| Materials and Methods   |       |           |
| Results   |       |           |
| Discussion  |       |           |
| Summary   |       |           |
| <br><b>Chapter 2. Production of Own-rooted Trees by Cutting Propagation</b> |       |           |
| <b>Section 1. Hardwood Cuttings</b>   | ----- | <b>31</b> |
| Introduction  |       |           |
| Materials and Methods   |       |           |

Results  
Discussion  
Summary

**Section 2. Softwood Cuttings ----- 40**

Introduction  
Materials and Methods  
Results  
Discussion  
Summary

**Chapter 3. Evaluation of Field Performance of Own-rooted Trees**

**Section 1. Evaluation of Micropropagated Trees**

**(I) Evaluation of Unpruned Trees ----- 49**

Introduction  
Materials and Methods  
Results and Discussion  
Summary

**(II) Long-term Evaluation of Pruned Trees ----- 55**

Introduction  
Materials and Methods  
Results  
Discussion  
Summary

**Section 2. Evaluation of Trees Propagated by Cuttings ----- 64**

Introduction  
Materials and Methods  
Results and Discussion  
Summary

**Conclusions and Prospects ----- 71**

**Literature Cited ----- 73**

**Publication List ----- 81**

**Acknowledgements ----- 83**

## **General Introduction**

Own-rooted fruit trees are rarely used in orchards, except for fig trees and blueberry plants, which are easy-to-root fruit species. Recently, the development in micropropagation of difficult-to-root fruit species has been expected to produce own-rooted trees, because the micropropagation would avoid raising rootstock and grafting (budding) scion, which are time-consuming and cumbersome operations. The use of micropropagation also makes it possible to rapidly propagate the clonal rootstocks with valuable traits, such as dwarfing effect and salt-, drought-, and water resistances, which are often difficult to propagate by cutting. Furthermore, if own-rooted fruit trees have such valuable traits, cumbersome grafting will be avoided. Therefore, the micropropagation of many fruit trees and rootstocks have been developed worldwide by many scientists (Bajaj, 1986), and there are some successful micropropagation laboratories producing fruit tree rootstocks in Italy and the United States (Howard, 1987). Howard (1987) pointed out, however, that the advantages of micropropagation were offset by the expensive facilities and by the fact that very small plants, less than 5 cm tall when transplanted, needed to increase in size between ten and twenty fold before being usable, and suggested that it was unlikely that micropropagation would be used to produce the finished rootstock, but rather to produce plants for stoolbeds and conventional hedges whose cuttings rooted well because they were rejuvenated.

### ***Cutting propagation of Japanese persimmon***

Propagation of Japanese persimmon cultivars by cuttings has so far proved to be very difficult (Kitagawa and Glucina, 1984), and few scientists tried it and succeeded in softwood cutting propagation (Machida and Fujii, 1969; Murata et al., 1983; Tukamoto et al., 1959). In all cases, etiolation of stock plants and blanching of cutting bases were necessary to obtain good rooting. These operations require a lot of time. Furthermore, long cuttings were used in these studies and only a limited number of cuttings could be obtained from a tree. Moreover, many cuttings died during their acclimatization to sunlight (Tukamoto et al., 1959), the number of roots per rooted cutting was few (Machida and Fujii, 1969), and only a few cuttings survived after rooting (Murata et al., 1983). Therefore, these propagation methods have never been used commercially, and there has been no report of field performance of the own-rooted trees propagated by cuttings.

Although there are many advantages in hardwood cutting propagation

(Macdonald, 1986), there have been no reports of hardwood cutting propagation of Japanese persimmon possibly because hardwood cuttings are more difficult to root than softwood cuttings (Gemma et al., 1983).

### ***Micropropagation of Japanese persimmon***

The potential use of micropropagation of Japanese persimmon was first indicated by Cooper and Cohen (1984). Tao and Sugiura (1992) described a series of works for micropropagation of Japanese persimmon in detail, and pointed out some problems that should be solved, such as rooting, acclimatization, and evaluation of field performance. The recent and main research for micropropagation was the improvements in rooting and acclimatization of rootstocks for Japanese persimmon (Ito et al., 1999; Kagami, 1995; Kagami et al., 1995; Matsumoto and Yamada, 1993). However, no improvements in rooting and acclimatization of Japanese persimmon cultivars have been reported probably because they were more difficult than those in rootstocks. The explants used in the rootstock studies were usually derived from the shoots from roots, which are thought to be in a juvenile phase and to be easy to micropropagate (Bonga, 1982).

Fumuro (1992) reported on the early field performance (four years) of micropropagated Japanese persimmon trees, but there have been no reports of the long-term evaluation. Zimmerman and Miller (1991) have indicated that the commercial benefits of micropropagated trees can only be determined after several years of field evaluation. Long-term studies have indicated the benefits in apple (Rosati and Gaggioli, 1989; Zimmerman and Miller, 1991; Larsen and Higgins, 1993), blueberry (El-Shiekh et al., 1996), and peach (Hammerschlag and Scorza, 1991).

The objectives of this study were to improve in rooting ability of microcuttings of Japanese persimmon cultivars, especially difficult-to-root cultivars, and to establish an efficient acclimatization method for rooted microcuttings (micropropagules). On the other hand, trials of propagation by softwood and hardwood cuttings of Japanese persimmon cultivars were made, with an application of an in vitro principle to ex vitro conditions. Moreover, the field performance of micropropagated Japanese persimmon trees was evaluated for a long term (pruned trees) as well as a short term (unpruned trees), in comparison with conventionally propagated trees (grafted on seedling stocks). Early field performance of trees propagated by cuttings was also evaluated in comparison with both micropropagated trees and conventionally propagated trees. The list of papers included in this study appears in the part before the last part of this paper.

## **Chapter 1.**

### **Production of Own-rooted Trees by Micropropagation**

#### **Section 1. Improvements in Rooting of Microcuttings**

##### **(I) Improvements by Using Shoots Subcultured In Vitro with BA**

###### **Introduction**

The development in micropropagation of Japanese persimmon was described in earlier parts of this paper. However, their application to commercial production has been limited partly because Japanese persimmon microcuttings generally are difficult to root. More than half of the cultivars tested by Fukui et al. (1992) had low rooting ability.

Zeatin [6-(4-hydroxy-3-methylbut-2-enylamino) purine] is the most effective of all cytokinins in micropropagating Japanese persimmon cultivars (Cooper and Cohen, 1984; Fukui et al., 1989), but it is expensive. On the other hand, 6-benzyladenine (BA), an inexpensive cytokinin was less effective in establishing and propagating Japanese persimmon cultivars' microshoots (Murayama et al., 1989; Sugiura et al., 1986). Tetsumura et al. (1991) reported that more cultivars were established in vitro with zeatin than with BA and that the shoots established and proliferated with zeatin showed lower rooting ability than those with BA.

In this subsection, the possible use of BA for shoot multiplication was explored to increase the rooting ability of shoots of 'Fuyu' and 'Hana Gosho', which are hard to establish in vitro with BA (Murayama et al., 1989; Tetsumura et al., 1991).

###### **Materials and Methods**

The methods of bud explant establishment, shoot proliferation and rooting were based on those reported by Tao and Sugiura (1992). Small buds, approx. 2 mm long, excised from dormant buds of 'Fuyu' and 'Hana Gosho' annual twigs collected in winter, were established in the modified Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) with 5  $\mu$  M zeatin and half-strength  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  [MS(1/2N)]. Shoots were proliferated by subculturing onto the fresh medium every 30 days. After subculturing 11 times, some shoots were transferred onto the same medium, but replacing zeatin with 20  $\mu$  M BA, and the others were transferred onto the MS medium with 5  $\mu$  M zeatin. Before rooting treatment, the shoots subcultured with BA were transferred onto the MS medium with 5  $\mu$  M zeatin to ensure shoot elongation and cultured for 30 days. Rooting was induced by dipping the basal ends of more than 1-cm

long shoots in 1.25 mM indole-butyric-acid (IBA) for 10 sec, after which they were placed onto half-strength MS(1/2N) medium without growth regulators. There were 21 to 84 shoots per rooting treatment. The percentage of shoots forming adventitious roots and the number of adventitious roots per rooted shoot were determined 40 days after the rooting treatment. The significant difference between the types of cytokinin used for the proliferation medium was analyzed by chi-square test.

All media contained 0.8% (w/v) agar (Wako Pure Chemicals Co, Tokyo) and 0.09 M sucrose. The pH of the media was adjusted to 5.7 before autoclaving at 121 °C and 120 kPa for 15 min. Three to four shoots were placed in each Erlenmeyer flask (100 ml) containing 30 ml of the proliferation medium or 50 ml of the rooting medium. Cultures were maintained at 28 °C under the 16 h/day photoperiod, except the dark incubation for initial 10 days of rooting treatment to ensure rooting, with a photon flux of  $60 \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  provided by cool-white fluorescent lamps.

## Results

At first, the shoots of 'Fuyu' and 'Hana Gosho' which were established and proliferated by zeatin stopped growing after transferring onto the MS(1/2N) medium supplemented with BA. They started growing vigorously again when subcultured onto the same medium continuously and showed a rosette type of growth like other Japanese persimmon cultivars' shoots proliferated with BA (Tao and Sugiura, 1992). On the elongation medium with zeatin, before rooting treatment, the shoots subcultured with BA were shorter than those with zeatin only, though there was no difference in the number of their leaves (data not presented).

During the initial period of subcultures with BA, regardless of their vigor in subculturing, the shoots of 'Fuyu' and 'Hana Gosho' rooted poorly after rooting treatment, like those subcultured with zeatin only (Fig. 1.1). After subculturing 17 times, however, the rooting ability of the shoots subcultured with BA increased with repeated subcultures. Finally, 63-65% of them rooted after subculturing 74-76 times (6.5 years). On the other hand, the rooting ability of the shoots of both cultivars subcultured with zeatin only was lower during the experiments. Less than 30% of them rooted constantly, and none of them rooted at some rooting experiments. The mean number of roots per rooted shoots subcultured with BA was 2.0-4.5 after subculturing more than 40 times, and most of the rooted shoots subcultured with zeatin were only one or two roots at all rooting experiments.

## Discussion



The phenomenon that the increasing rooting ability in the shoots of apple and Japanese pear achieved with successive subcultures was explained by progressive rejuvenation of these shoots with subcultures (Banno et al., 1989; Sriskandarah et al., 1982). Since the increasing rooting ability was achieved by subculturing with BA rather than zeatin in this study, BA might rejuvenate the in vitro Japanese persimmon cultivars' shoots more effectively than zeatin. However, an investigation of the first flowering of micropropagated plants

previously subcultured with BA should be necessary to confirm whether BA induced true rejuvenation in the shoots of Japanese persimmon cultivars. Tao et al. (1994) concluded that partial rejuvenation or reinvigoration, but not true rejuvenation, occurred to 'Jiro' shoots subcultured with zeatin.

Zeatin is very expensive cytokinin. Therefore, it is recommended that BA should be substituted for zeatin in the proliferation medium, if the Japanese persimmon cultivars' shoots needed to be micropropagated are able to proliferate on the medium with BA. In addition to the cost, BA would be more favorable for increasing the rooting ability of the shoots after successive subculturing.

In the next subsection, more improvements in rooting were suggested, but the

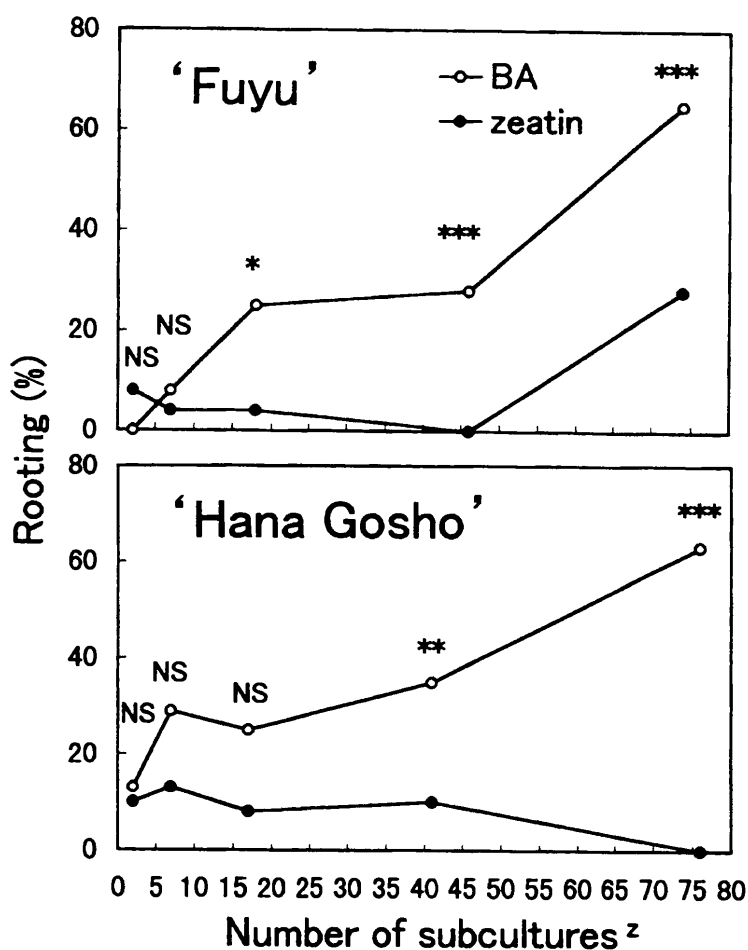


Fig. 1.1. Effects of the number of subcultures and the types of cytokinin used for proliferation medium on the subsequent rooting of two Japanese persimmon cultivars' shoots. NS, \*, \*\*, \*\*\* Nonsignificant or significant at  $P < 0.05$ , 0.01, 0.001, respectively. <sup>z</sup> After establishing and subculturing 11 times on the MS(1/2N) medium supplemented with zeatin.

method indicated there needed to use in vitro roots. To begin with, therefore, it must be important to induce rooting of difficult-to-root cultivars. The method indicated here would be an important technique for improving in rooting of difficult-to-root cultivars.

### **Summary**

After establishing and proliferating onto the modified MS medium supplemented with zeatin, the in vitro shoots of two Japanese persimmon cultivars were cultured on the same medium with BA substituted for zeatin. Although the shoots of 'Fuyu' and 'Hana Goshō', which are hard to establish in vitro with BA, stopped growing after transferring to the medium with BA, they started growing vigorously again when subcultured continuously with BA. During the initial period of subculturing with BA, they rooted poorly after rooting treatment, like those subcultured with zeatin only. After subculturing 17 times, however, the rooting ability of the shoots subcultured with BA increased with repeated subcultures. After 74-76 times, finally, the shoots subcultured with BA showed a substantially higher rooting percentage (63-65%) than those with zeatin (less than 30%). The mean number of the roots per rooted shoots subcultured with BA was 2.0-4.5 after subculturing more than 40 times and most of the rooted shoots subcultured with zeatin only had one or two roots at all the rooting experiments.

## **(II) Improvements by Using Shoots Differentiated from In Vitro Roots**

### **Introduction**

In the previous subsection, long-term subculture with BA improved the rooting ability in some cultivars that rooted poorly when subcultured with zeatin. However, 74-76 subcultures (6.5 years) were required before satisfactory rooting percentages could be obtained.

In several apple cultivars, adventitious shoots regenerated from root cuttings readily formed their own roots (Robinson and Schwabe, 1977). Furukawa et al. (1990) reported that shoots regenerated from root cultures of *Eustoma grandiflorum* (Griseb.) Schinners rooted easily and that culture of roots seemed to be useful for micropropagation. Similarly, if adventitious microshoots regenerated from roots of Japanese persimmon cultivars have higher rooting ability, they will be a better source of stock shoots for micropropagation than the original shoots. However, there has been no report of in vitro shoot regeneration from roots of Japanese persimmon cultivars, and the rooting and growth of shoots regenerated from in vitro culture of roots of woody plants has not been investigated. The shoots that arise on root cuttings, when used as softwood cuttings, tend to root more readily than cuttings taken from other parts of the tree (Del Tredici, 1995).

In preliminary experiments, the induction of in vitro shoot regeneration from roots of some Japanese persimmon cultivars was successfully achieved, but the rate of such shoot formation was low. Hence, the objective of the first part of this subsection was to obtain high-frequency shoot regeneration from roots of 'Jiro' Japanese persimmon by investigating the influence of growth regulators, length of root explants, and whether root explants had an apical meristem on shoot regeneration from roots. The objective of the second part of this subsection was to explore the organogenetic capacity of the roots of four Japanese persimmon cultivars and to compare the rooting potential and the growth of shoots regenerated from them with those originating from shoot tips. Furthermore, their histology during morphogenesis was examined, and trees regenerated from roots were planted in an orchard in order to be investigated on their precocity and possible variation.

### ***(a) High-frequency Shoot Differentiation from In Vitro Roots***

#### **Materials and Methods**

*General procedure.* The methods for bud explant establishment, shoot proliferation, and rooting were based on those reported by Tao and Sugiura (1992a). Small buds, approx. 2 mm long, excised from dormant buds of 'Jiro' Japanese persimmon were established in modified MS salts plus vitamins with 5  $\mu$  M zeatin and half-strength  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$ . Shoots were maintained for over two years by subdividing into nodal cuttings and transferring onto full strength MS medium with 5  $\mu$  M zeatin every 30 days. Rooting was induced by dipping the basal ends of 1- to 2-cm-long shoots in 1.25 mM IBA for 10 sec, after which the shoots were placed on half-strength MS with half-strength N and without growth regulators. After 40 days in the rooting medium, lateral roots were removed from primary roots originating from the microshoots. These primary roots were excised from their shoots and used for regeneration studies.

All media contained 0.8% (w/v) agar (Wako Pure Chemicals Co., Tokyo) and 0.09 M sucrose. The pH of the media was adjusted to 5.7 before autoclaving at 121 °C and 120 kPa for 15 min. Two to four root segments were placed in each Erlenmeyer flask (100ml) containing 30 ml of the differentiation medium. Cultures were maintained at 28 °C under a 16-h/day photoperiod with a photon flux of 60  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  provided by cool-white fluorescent lamps.

*Growth regulators.* Terminal root segments, including the root apex, were cut to  $4.5 \pm 1.5$  cm long and cultured on MS medium supplemented with either zeatin, *N*-phenyl-*N'*-(2-chloro-4-pyridyl) urea (CPPU), BA, or 2-isopentenyladenine (2iP) (see Table 1.1. for concentrations) in combination with 0.01  $\mu$  M indole-3-acetic acid (IAA), or on MS medium containing IAA, IBA, 1-naphthalene-acetic acid (NAA), or 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.1, 0.01, and 0.001  $\mu$  M in combination with 10  $\mu$  M zeatin. There were 18 to 20 roots per treatment.

*Root length and root apex.* Root segments with or without a root apex were cut to either 1, 2, 3 to 4 to 6 cm long and cultured on MS medium supplemented with 10  $\mu$  M zeatin and 0.01  $\mu$  M IAA. There were 18 to 20 roots per treatment.

*Analytical methods.* Each experiment was conducted twice. The percentage of root segments forming adventitious shoots was determined after 60 days of culture. The number of root segments forming more than five adventitious shoots also was recorded. Percentage data were subjected to arcsin transformation and then significance of treatment effects was determined by using analysis of variance (ANOVA). Variation

among treatment means was analyzed by using Tukey's (1953) procedure.

## Results and Discussion

After 20 days of culture, adventitious shoots differentiated spontaneously from roots (Fig. 1.2). The shoots arose directly from root tissue, not from callus, in the same way as previously reported in red raspberry (*Rubus idaeus* L.) (Borgman and Mudge, 1986) and prairie gentian [*Eustoma grandiflorum* (Griseb.) Schinners] (Furukawa et al., 1990).

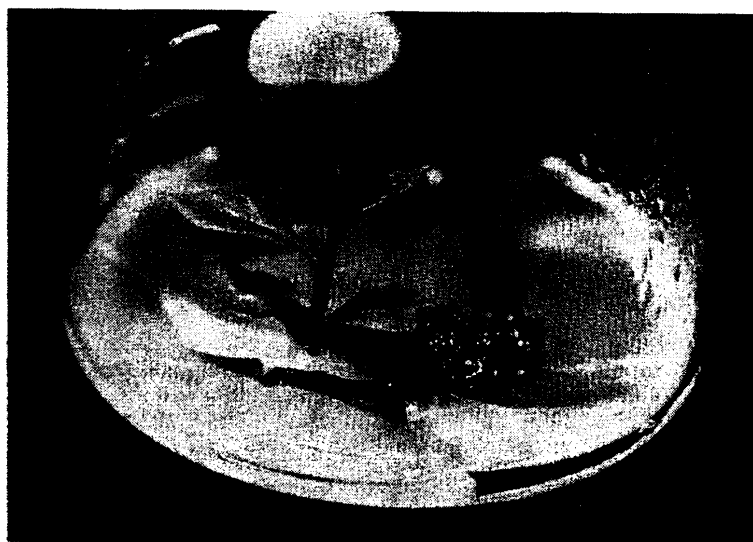


Fig. 1.2. Adventitious shoot formation from root culture of 'Jiro' Japanese persimmon on solidified MS medium supplemented with  $10^{-5}$  M zeatin and  $10^{-8}$  M IAA after 40 days of culture.

**Growth regulators.** All the cytokinins tested induced adventitious shoot formation on roots (Table 1.1). When no cytokinin was present in the medium, adventitious shoot formation did not occur. In comparing various kinds of cytokinins at several concentrations in combination with  $0.01 \mu\text{M}$  IAA, the best result in terms of percent root segments forming adventitious shoot was obtained with  $10 \mu\text{M}$  zeatin, although the effectiveness of this treatment did not differ significantly from that of several others. This effect of zeatin is similar to that reported for adventitious bud formation from callus (Tao and Sugiura, 1992b) and shoot tip cultures (Cooper and Cohen, 1984; Tetsumura et al., 1991) of Japanese persimmon cultivars. The concentrations of CPPU were set an order of magnitude lower than those of the other cytokinins, because several substituted pyridyl phenylurea compounds have been demonstrated to stimulate in vitro meristem and shoot formation at unusually low concentrations (Mok et al., 1987). The two best CPPU concentrations tested for adventitious shoot formation were lower than similarly effective ones for other cytokinins. The natural cytokinin 2iP effectively induced adventitious shoots from roots and also stimulated shoot elongation (Sugiura et al., 1986), but it had little effect on adventitious bud formation from callus culture (Tao and Sugiura, 1992b). BA mostly

was less effective than the other cytokinins. If the shoots regenerated from roots are wanted for an experiment, 100  $\mu\text{M}$  zeatin and 10  $\mu\text{M}$  CPPU also will be useful because they can induce many adventitious shoots.

Table 1.1. Effect of various types of cytokinins at several concentrations on adventitious shoot formation from roots of 'Jiro' Japanese persimmon. All media contained 0.01  $\mu\text{M}$  IAA.

| Cytokinin | Concn<br>( $\mu\text{M}$ ) | Percent root segments<br>forming adventitious shoots |                       |
|-----------|----------------------------|--|-----------------------|
|           |                            | Total  | More than five shoots |
| —         | 0                          | 0 a <sup>2</sup>                                     | 0 a                   |
| Zeatin    | 1                          | 35 b   | 3 ab                  |
|           | 10                         | 85 d   | 48 d                  |
|           | 100                        | 53 bc  | 43 d                  |
|           | 100                        | 53 bc  | 43 d                  |
| CPPU      | 0.1                        | 0 a  | 0 a                   |
|           | 1                          | 76 cd  | 34 cd                 |
|           | 10                         | 50 bc  | 15 a-d                |
| BA        | 1                          | 0 a  | 0 a                   |
|           | 10                         | 33 b   | 5 a-c                 |
|           | 100                        | 0 a  | 0 a                   |
| 2iP       | 1                          | 3 a  | 0 a                   |
|           | 10                         | 63 cd  | 30 b-d                |
|           | 100                        | 70 cd  | 55 d                  |

<sup>2</sup>Mean separation in columns by Tukey's test ( $P < 0.05$ ). Values are means of two replications consisting of 18 to 20 roots each.

Auxin was not essential to produce adventitious shoots on roots, and at higher concentrations, especially 2,4-D, stimulated excessive growth of callus and inhibited adventitious shoot formation (data not presented).

*Root length and root apex.* According to ANOVA, there was no significant effect of the presence of a root apical meristem and no significant interaction between it and root length ( $P < 0.05$ ). The percentage of root segments forming adventitious shoots

increased with increasing segment length (Table 1.2), and the regression equation between them [ $y (\%) = -8.72 + 22.2x (\text{length})$ ] showed a high correlation ( $P < 0.001$ ,  $r^2 = 0.90$ ). Almost all of the 4- to 6-cm root segments formed adventitious shoots. In preliminary experiments, roots were cut into small pieces and placed them on the medium. Adventitious shoot formation scarcely occurred. The percentage of 3- to 4-cm root segments forming many adventitious shoots was similar to that for 4- to 6-cm segments. Yamada et al. (1988) showed that root length was important for the regeneration of Japanese persimmon from root cuttings.

Table 1.2. Effect of root length on adventitious shoot formation from roots of 'Jiro'

Japanese persimmon. All media contained 10  $\mu\text{M}$  zeatin and 0.01  $\mu\text{M}$  IAA.

| Root length<br>(cm) | Percent root segments forming adventitious shoots |                       |
|---------------------|---|-----------------------|
|                     | Total   | More than five shoots |
| 1                   | 16 a <sup>2</sup>                                 | 1 a                   |
| 2                   | 26 a  | 6 a                   |
| 3-4                 | 81 b  | 42 b                  |
| 4-6                 | 98 c  | 54 b                  |

<sup>2</sup>Mean separation in columns by Tukey's test ( $P < 0.05$ ). Values are means of four replications consisting of 18 to 20 roots each.

It seems likely that the high organogenetic potential of roots of Japanese persimmon 'Jiro', i.e., the high-frequency shoot regeneration from roots, relates to juvenility, for, as Tamura et al. (1992) have suggested, rejuvenated Japanese persimmon callus has a high capacity for adventitious bud formation, while Bonga (1982) has reported that the roots often retain their juvenility. If shoots regenerated from roots succeed in retaining juvenility, they can easily root and be micropropagated.

## Summary

Microshoots of 'Jiro' Japanese persimmon rooted in vitro. The roots were excised and cultured on solidified Murashige and Skoog medium. After 20 days of culture, adventitious shoots formed spontaneously and directly from the roots. Of all the tested cytokinins, 10  $\mu\text{M}$  zeatin in combination with 0.01  $\mu\text{M}$  IAA was the most

effective in stimulating production of adventitious shoots. CPPU and 2iP also were effective cytokinins. Addition of a high concentration of auxin, especially 2,4-D, to the medium inhibited adventitious shoot formation. The percentage of root segments forming adventitious shoots increased with increasing segment length. Almost all of the longest roots (4 to 6 cm) formed adventitious shoots.



## ***(b) Characteristics of Shoots Differentiated from In Vitro Roots***

### **Materials and Methods**

*Original plant materials.* Micropropagated plantlets of four cultivars derived from in vitro shoot cultures of Japanese persimmon were used as source material. The rooting ability of 'Jiro' is high, that of 'Hiratanenashi' moderate, and that of 'Hana Goshō' and 'Fuyu' low. Shoot cultures from dormant buds were established as described by Tao and Sugiura (1992a). Shoot multiplication cultures were maintained for more than four years by subculturing at 30-day intervals (more than 50 cultures) on MS salts plus vitamins with 5  $\mu$  M zeatin (multiplication medium). Rooting was induced by dipping the basal ends of shoots approx. 1 cm long in 1.25 mM IBA in 50% ethanol for 10 sec, after which the shoots were placed on half-strength MS with half the normal strength of nitrates and without growth regulators (rooting medium). After 40 days in the rooting medium, lateral roots were removed from the primary roots originating from the shoots. These primary roots were excised from the shoots, measured, and used for the regeneration experiment.

*Regeneration from roots.* Root segments were cultured on MS medium supplemented with 10  $\mu$  M zeatin and 0.01  $\mu$  M IAA (regeneration medium) to induce adventitious shoots. The percentage of root segments forming adventitious shoots was determined after 60 days of culture, and the number that formed more than five adventitious shoots was also recorded. There were 10 to 32 root segments per cultivar, and the experiment was conducted four times.

*Histology.* After 0-30 days on the regeneration medium, small pieces (5-10 mm) of 'Jiro' roots were fixed in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Tissues were dehydrated in a graded ethanol series, and then infiltrated with acrytron E (Mitsubishi Rayon Co., Tokyo). Sections (4  $\mu$  m) were cut with a glass knife, stained in 0.05% toluidene blue O, and observed by light microscopy.

*Rooting and growth.* Adventitious shoots were excised from the roots, adjusted to apical buds containing two leaves, and cultured once on multiplication medium. The number of shoots, the height of the tallest shoot, and the number of leaves were recorded after 30 days of culture. The rooting treatment, as stated above, was then carried out with shoots approx. 1 cm long. The percentage of shoots forming adventitious roots was examined at 10-day intervals, and the number of adventitious roots per rooted shoot was determined at the end of the culture period. Ten shoots were used per cultivar, and the experiment was conducted four times. The experiments were also conducted on mother stock shoots that originated from shoot tips and produced

adventitious roots that were used for regeneration experiments.

The rooted shoots were potted and acclimatized according to the next section. After the rooting medium was rinsed off in sterile water, rooted shoots were planted in 60 × 60-mm pots filled with fine vermiculite, and the medium was saturated with half-strength liquid MS salts with half the normal strength of nitrates and without sucrose, in 75 × 100-mm polycarbonate vessels that were autoclaved. Between 30 and 60 days after planting, the cover was gradually removed from the vessel. Survival percentage, plant height, the number of new leaves that developed after potting, and the dry weight of top were recorded 30 days after acclimatization. Since the acclimatization experiment was carried out after the rooting experiment, the number of plants ranged from 2 to 10 depending on the percentages of rooting. The experiment was conducted four times.

*Changes in rooting ability during subculture.* Shoots regenerated from ‘Jiro’ roots were subcultured 10 times on the multiplication medium, and changes in their rooting ability during subcultures were evaluated. At each subculture, 20 shoots were used for the rooting test, and the rest were planted on the multiplication medium. The experiment was conducted twice, and the mother shoots were also used for the same rooting test. Rooting was recorded after 20 days of the rooting treatment.

Throughout the course of the experiments, all of the media contained 0.8% (w/v) agar (Wako Pure Chemicals Co., Tokyo) and 0.09 M sucrose. The pH of the media was adjusted to 5.7 before autoclaving at 121 °C and 120 kPa for 15 min. Two root segments or three to four shoots were placed in an Erlenmeyer flask (100 mL) containing 30 mL medium. However, the volume of the rooting medium was 50 mL. Cultures were maintained at 28 °C under a 16-h photoperiod, except for dark incubation for the initial 10 days of the rooting treatment (Murayama et al., 1989) and continuous lighting for acclimatization according to the next section, with a photon flux of  $60 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  provided by cool-white fluorescent lamps.

*Field performance.* After acclimatization, the plantlets regenerated from roots and those that originated from shoot tips of both ‘Jiro’ and ‘Hiratanenashi’ were transplanted to 20 × 20-cm clay pots, grown outdoors for a year, and then established in clay loam at the orchard of the Experimental Farm of Kyoto University. One-year-old nursery trees grafted on seedlings were also established. Nine trees per treatment per cultivar were planted in randomized block design with three blocks. Flowering was recorded in May. In addition to these trees, 30 trees regenerated from ‘Jiro’ roots were planted in the same orchard to determine if there was variation in the regenerants from roots.

*Statistical analysis.* All data were subjected to ANOVA and cultivar means were separated by Duncan's multiple range test. Percentage data were subjected to arcsin transformation before analysis.

## Results

*Regeneration from roots.* Root segments of the four cultivars tested produced adventitious shoots (Table 1.3). Shoots regeneration was more prolific from 'Jiro' roots than from the other cultivars, because about one half of the root segments produced more than five adventitious shoots. Comparison of regeneration capacity of roots among cultivars was confounded by the greater tendency of longest root segments to form adventitious shoots, which was

Table 1.3. Length of primary roots induced on in vitro shoots 40 days after 1.25 mM IBA treatment and subsequent adventitious shoot formation from root segments of four Japanese persimmon cultivars.

| Cultivar      | Mean length<br>of root segments<br>(cm) | Root segments forming<br>adventitious shoots (%) |                       |
|---------------|---|--|-----------------------|
|               |   | Total  | More than five shoots |
| Fuyu          | 4.5 ab <sup>2</sup>                     | 51 b   | 12 a                  |
| Hana Gosho    | 6.0 c                                   | 31 a   | 2 a                   |
| Hiratanenashi | 3.5 a                                   | 55 b   | 5 a                   |
| Jiro          | 4.4 b                                   | 85 c   | 45 b                  |

<sup>2</sup>Mean separation within columns by Duncan's multiple range test ( $P < 0.05$ ).

Values are means of four replications consisting of 10 to 32 roots each.

shown in the first part of this subsection, and the mean length of the root segments varied with cultivar. However, the roots of 'Hana Gosho' demonstrated the lowest regeneration capacity of all of the cultivars tested, since its percentage of adventitious shoot formation was the lowest, although its mean root length was the longest.

*Histology.* Adventitious roots, which were tetra- or pentarch, consisted of a single epidermal layer, a parenchymatous cortex containing starch grains, a single-layered endodermis and pericycle, and a central vascular cylinder without pith (Fig. 1.3A). On regeneration medium, the roots began to show secondary thickening; then the meristematic tissue appeared in a proliferated pericycle below the protoxylem poles between the phloem bundles (Fig. 1.3B), expanded centrifugally, and became an adventitious shoot primordium (ASP) (Fig. 1.3C). When the ASP was about to emerge, a procambial strand elongated to the ASP from the vascular cambium of the root (Fig. 1.3D). Finally, the ASP differentiated into a shoot bud with a distinct shoot apex and leaf primordium, and emerged from the root (Fig. 1.3E).

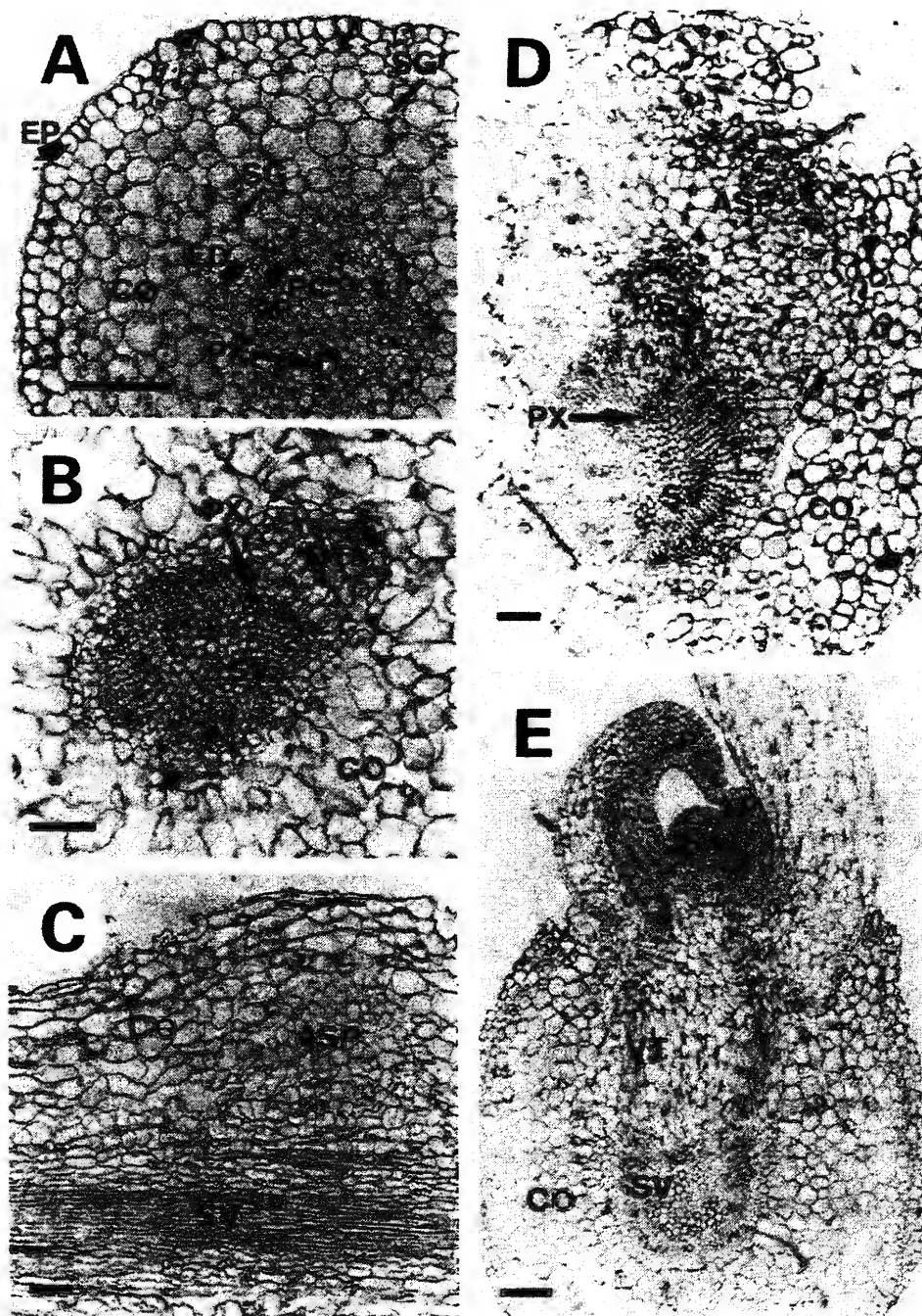


Fig. 1.3. (A, B, D, and E) Transverse and (C) longitudinal sections of 'Jiro' Japanese persimmon roots cultured in vitro. (A) An adventitious root before culture on regeneration medium. Most of the cortical cells contain starch grains. (B) Early stage of secondary thickening after 15 days on regeneration medium. Meristematic tissue appears in the proliferated pericycle. (C) A protuberance on the root after 18 days on regeneration medium. An adventitious shoot primordium (ASP) is expanding centrifugally. The endodermis has degenerated. (D) The ASP with a procambial strand after 20 days on regeneration medium. The cortex and epidermis near the ASP have degenerated. (E) A well-developed shoot bud with a few leaf primordia enclosing a shoot apex. A normal vascular system has been completed from the shoot bud to the main root vascular cylinder. Abbreviations: cortex (CO), endodermis (ED), epidermis (EP), leaf primordium (LP), meristematic tissue (MT), pericycle (PC), procambial strand (PS), protoxylem (PX), shoot apex (SA), starch grain (SG), secondary vascular tissue (SV), vascular tissue (VT). All scale bars = 0.1 mm.

### *Rooting and growth.*

Shoots regenerated from roots seemed to grow more vigorously than those that originated from shoot tips after 30 days on multiplication medium, and there were significant differences in the number of leaves of 'Jiro' and the number of shoots of 'Hana Gosho' (Table 1.4). However, there were no significant differences in the height of the tallest shoots within cultivars. No variants were observed among the shoots regenerated from roots of the four cultivars.

Shoots regenerated from roots rooted better than those that originated from shoot tips and had been subcultured more than 50 times (Fig. 1.4). For the low-rooting-ability cultivars, Fuyu and Hana Gosho, the differences in rooting

Table 1.4. Comparison of the growth of shoots regenerated from roots (R) with those originating from shoot tips (S) of four Japanese persimmon cultivars after 30 days on multiplication medium.

| Cultivar      | Origin | No. of shoots   | No. of leaves |
|---------------|--------|-----------------|---------------|
| Fuyu          | R      | 2.2             | 5.8           |
|               | S      | 2.0             | 5.8           |
|               |        | NS <sup>2</sup> | NS            |
| Hana Gosho    | R      | 2.8             | 6.9           |
|               | S      | 2.2             | 7.0           |
|               |        | *               | NS            |
| Hiratanenashi | R      | 2.9             | 4.6           |
|               | S      | 3.1             | 4.3           |
|               |        | NS              | NS            |
| Jiro          | R      | 2.8             | 6.2           |
|               | S      | 2.8             | 5.0           |
|               |        | NS              | *             |

<sup>2</sup> NS, Nonsignificant or significant difference between origins within cultivars at  $P < 0.05$  by ANOVA. Values are means of four replications consisting of 10 shoots each.

percentages between the two types of shoots were significant after 20 or 30 days of rooting treatment, and the rooting percentages of shoots regenerated from roots were twice as high as those of shoots that originated from shoot tips after 40 days. Even for the moderate-rooting-ability cultivar, Hiratanenashi, the rooting percentage of shoots regenerated from roots was significantly higher than that of the mother shoots after 40 days. Because of the high rooting ability of the mother shoots of 'Jiro', the differences in rooting percentage were not significant on the 40th day. However, shoots regenerated from roots rooted 10 days earlier than those that originated from shoot tips. No

differences in the number of adventitious roots per rooted shoot were significant except for 'Jiro', in which shoots from shoot tips formed 2.1 adventitious roots, while those from roots formed 4.7 roots ( $P < 0.05$ ).

After potting, the rooted shoots from 'Jiro' roots grew better than those that originated from shoot tips (Table 1.5). Although Japanese persimmon shoots tend to stop growing during rooting, 53% of the shoots regenerated from 'Jiro' roots started to grow vs. only 28% of those that originated from shoot tips. Rooted shoots regenerated from 'Hiratanenashi' roots acclimated more easily than those that originated from shoot tips. As a result, two-thirds of the regenerated shoots subjected to the rooting treatment survived after potting vs. only one-third of those from shoot tips. After the rooting treatment, survival in the pots was twice as great for shoots regenerated from roots of the cultivars with low rooting ability as for those that originated from shoot tips. However, no differences were observed in the survival percentages of rooted shoots and the growth after potting.

*Changes in rooting ability during subculture.* Although there was neither a linear nor a quadratic relationship between the rooting percentage of shoots regenerated from roots and the number of subcultures, the mean rooting percentage over ten

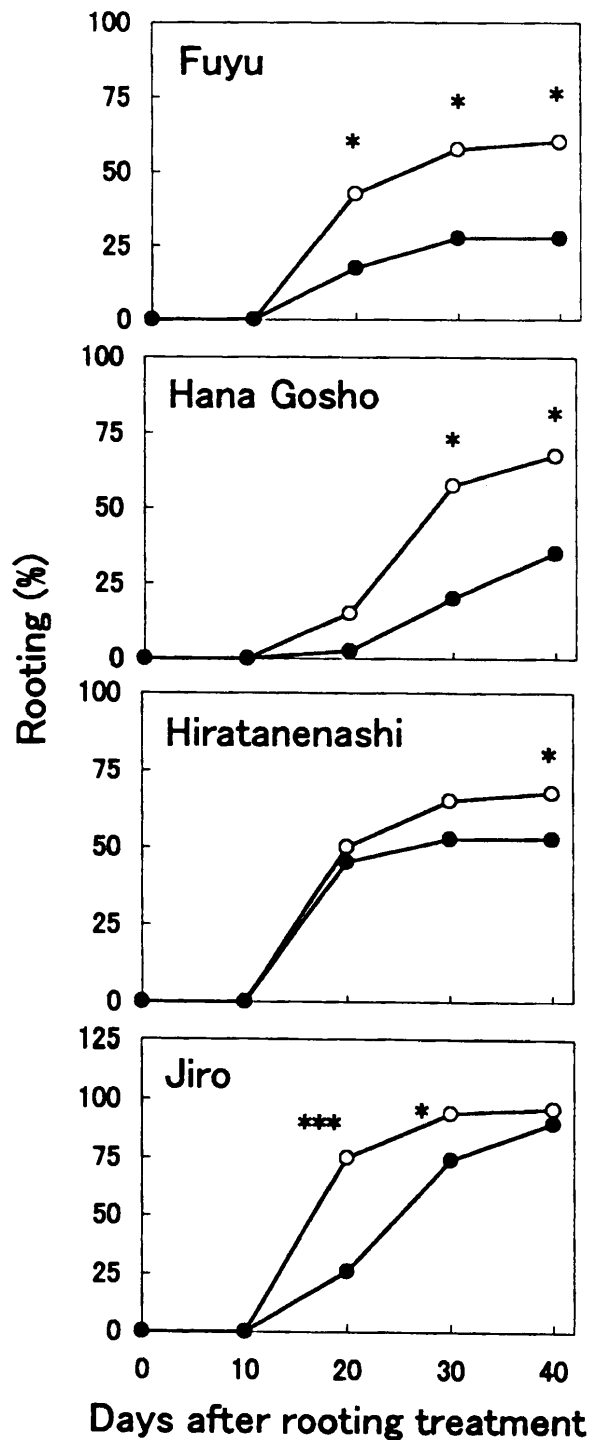


Fig. 1.4. Rooting percentage of shoots regenerated from roots (○) and those that originated from shoot tips (●) of four Japanese persimmon cultivars. Within cultivars and days, asterisks indicate significant difference at  $P < 0.005$  (\*\*\*) or 0.05 (\*) by ANOVA. Values are means of four replications consisting of 10 shoots each.

Table 1.5. Comparison of survival and growth of plantlets regenerated from roots (R) or shoot tips (S) of four Japanese persimmon cultivars 30 days after acclimatization.

| Cultivar      | Origin | Survival (%)                           |                     | Growth of surviving plantlets |                 |                          |
|---------------|--------|--|---------------------|-------------------------------|-----------------|--------------------------|
|               |        | Per shoot<br>with rooting<br>treatment | Per rooted<br>shoot | Plant<br>height<br>(cm)       | Leaves<br>(no.) | Top dry<br>weight<br>(g) |
| Fuyu          | R      | 48                                     | 78                  | 27.7                          | 11.4            | 3.6                      |
|               | S      | 23                                     | 83                  | 28.2                          | 11.4            | 2.9                      |
|               |        | * <sup>2</sup>                         | NS                  | NS                            | NS              | NS                       |
| Hana Gosho    | R      | 60                                     | 90                  | 24.5                          | 13.5            | 3.2                      |
|               | S      | 30                                     | 96                  | 24.2                          | 12.9            | 3.7                      |
|               |        | *                                      | NS                  | NS                            | NS              | NS                       |
| Hiratanenashi | R      | 63                                     | 92                  | 26.6                          | 14.0            | 3.3                      |
|               | S      | 33                                     | 62                  | 26.0                          | 13.3            | 2.4                      |
|               |        | *                                      | *                   | NS                            | NS              | *                        |
| Jiro          | R      | 90                                     | 98                  | 31.0                          | 15.1            | 3.1                      |
|               | S      | 78                                     | 96                  | 25.2                          | 12.3            | 1.8                      |
|               |        | NS                                     | NS                  | *                             | *               | *                        |

<sup>2</sup> NS,\* Nonsignificant or significant difference between origins within cultivars at  $P < 0.05$  by ANOVA. Values are means of four replications consisting of 2 to 10 plantlets each.

subcultures was significantly ( $P < 0.01$ ) higher than that of the shoots that originated from shoots tips (85% vs. 32%).

*Field performance.* Regardless of tree type, 50% to 66% of 'Jiro' trees flowered in the first year of establishment, and all flowered in the third year. None of the 'Hiratanenashi' trees flowered in the first year, but 33% of those micropropagated from shoot tips flowered in the second year and all flowered in the fourth year. The bark, leaves, flowers, and fruits of the two types of micropropagated trees were true-to-type, and no variants were observed among the 30 trees regenerated from 'Jiro' roots.

## Discussion

As previously reported for *Prunus* hybrid *incisa*  $\times$  *serrula* (Druart, 1980) and

apple root stock (*Malus prunifolia* Borkh.) (Masuda et al., 1994), roots of four Japanese persimmon cultivars formed adventitious shoots in vitro without difficulty. Because of the high organogenetic capacity, many regenerated shoots could be obtained from roots even of 'Fuyu', which is considered to be a difficult-to-root cultivar. Regarding 'Hana Gosho', however, the shoots were difficult-to-root and the roots had a lower regeneration capacity, so that we had to prepare more mother shoots to obtain enough roots to investigate the characteristics of the shoots that regenerated from them. Shoots regenerated from roots over ten subcultures maintained their ability to root. Therefore, once adventitious shoots have formed on roots, a great number of shoots with higher rooting ability can be obtained after multiplying them on the proliferation medium, even for more recalcitrant cultivars such as 'Hana Gosho'.

As for Japanese persimmon, adventitious shoots seem to form more easily on roots than on callus derived from primordial leaves. Although Tao and Sugiura (1992b) reported that the callus of 'Fuyu', 'Hana Gosho', and 'Hiratanenashi' did not form any adventitious buds, the root segments of these cultivars did so. Such high organogenetic potential of roots may be related to juvenility, which was discussed in the first part of this subsection.

In this study, shoots regenerated from roots, which are thought to be in a juvenile phase, had a higher rooting ability than their mother shoots. Incidentally, as stated in the previous subsection, the phenomenon of increasing rooting ability of shoots from adult plants with successive subcultures may be explained by the progressive rejuvenation of these shoots. The number of subcultures required for rejuvenation varies with species and cultivar (Banno et al., 1989; Zimmerman and Fordham, 1985), and is large in some fruit trees: 31 in the 'Red Delicious' apple (Sriskandarajah et al., 1982) and 38 in the 'Jiro' Japanese persimmon (Tao et al., 1994). In this study, the shoots from shoot tip cultures were thought to be rejuvenated to some extent because they were subcultured more than 50 times. However, through adventitious shoot formation on roots, which might have caused greater rejuvenation, the shoots with higher rooting ability than these rejuvenated shoots were obtained in this study.

After potting, the rooted shoots regenerated from roots survived and grew as well as or even better than those that originated from shoot tips. The earlier rooting of the regenerated shoots of 'Jiro' apparently led to a quicker recovery from damage by the rooting treatment. As a result, they developed more new leaves while cultured on the rooting medium and grew better after potting. Furthermore, the fact that rooted shoots from 'Hiratanenashi' roots were superior in survival and top dry weight after potting to those that originated from shoot tips might be related to juvenility.



In the first part of this subsection, adventitious shoots were observed arising directly from the root tissue, not from callus, while histological investigations in this study revealed that their origin was the pericycle tissue, as in *Citrus aurantifolia* (Christm.) Swing (Bhat et al., 1992). Direct organogenesis is preferable in the micropropagation industry in order to avoid producing somaclonal variants (Islam et al., 1992). Bhat et al. (1992) have inferred that pericycle cells are stable, indicating no apparent aberrations in the chromosomes of plants regenerated from roots. Even after passing through a callus phase, plants regenerated from roots seemed to be true-to-type (Jones et al., 1984; Srivastava et al., 1985). Although the sample size was small, the field performance of trees regenerated from roots in the present experiments also showed no variants. Therefore, this scheme may have commercial application.

Precocity is usually inconsistent with rejuvenation, which is assumed to cause a higher rooting ability of shoots regenerated from roots. After field establishment, however, the trees regenerated from roots began to flower as early as those micropropagated from shoot tips or grafted onto seedlings. Hence, the phase of the shoots regenerated from roots was not completely, but only partially rejuvenated, as reported by Tao et al. (1994).

In conclusion, shoots regenerated from roots appear to be useful for micropropagation of the Japanese persimmon, for both difficult-to-root and easy-to-root cultivars. This may facilitate the adoption of micropropagated trees in some commercial orchards.

## Summary

When cultured in vitro, the roots of four Japanese persimmon cultivars formed adventitious shoots on MS medium with 10  $\mu$  M zeatin and 0.01  $\mu$  M IAA, although their organogenetic capacities varied. Histological study revealed that the origin of the adventitious shoots was the pericycle. The regenerated shoots grew well on the shoot proliferation medium (MS with 5  $\mu$  M zeatin). Final rooting percentages of shoots regenerated from roots of three of the four cultivars were higher than those of shoots that originated from shoot tips and that had been subcultured more than 50 times. The shoots regenerated from 'Jiro' roots rooted 10 days earlier, had more roots than those from shoot tips, and maintained higher rooting ability over ten subcultures. Rooted 'Hiratanenashi' shoots regenerated from roots survived better after acclimatization than those from shoot tips. No obvious variants were observed either in vitro or in the field. The trees regenerated from roots flowered within 4 years. These findings suggest that partial rather than true rejuvenation was responsible for both the early flowering and the

juvenile characteristics, i.e., the enhanced rooting ability, observed in the regenerated plants.

## Section 2. Improvements in Acclimatization of Micropropagules

### Introduction

The in vitro rooted shoots of Japanese persimmon cultivars were difficult to acclimatize to field conditions (Cooper and Cohen, 1984; Murayama et al., 1989), and there have been no report of the improvements in their acclimatization, except for the method suggested by Tao and Sugiura (1992a). However, they concluded that the further improvements in acclimatization methods were necessary because a quarter to half of the rooted microcuttings (micropropagules) could be acclimatized to field conditions.

The objective of this section was to investigate the optimum conditions, especially of light and temperature, for micropropagules during acclimatization. Furthermore, acclimatized micropropagules were planted in pots or in an outdoor nursery in order to be investigated on the growth after being transferred outside.

### Materials and Methods

Microshoots were originated from dormant buds of one-year-old shoots taken from 'Nishimurawase' trees in the Shiga Agricultural Experimental Station. The methods of bud explant establishment, shoot proliferation, shoot elongation and rooting were based on those reported by Tetsumura et al. (1991), except for 20  $\mu$ M BA for the establishment and the proliferation cytokinin and 5  $\mu$ M zeatin for the elongation cytokinin.

#### *Effect of temperature and photoperiod on acclimatization (Expt. 1)*

The microshoots that were over 15 mm in length and had more than two leaves were conducted with the rooting treatment. The rooted shoots were used for the experiments 20 days after the rooting treatment. After the rooting medium was rinsed off in sterile water, they were each planted in 60 × 60-mm Jiffy round pots (Jiffy-Pot Products Co. of Japan, Ltd., Yokohama) filled with fine vermiculite (80 ml), and the medium was saturated with Hyponex 5-10-5 solution (Hyponex Japan Co. Ltd., Osaka) at 2 ml/L, in 75 × 100-mm polycarbonate vessels. The pots, the medium, and the vessels were autoclaved before the planting. The micropropagules expanding two new leaves in the vessels were acclimatized to the air by gradual removal of the vessels' covers, and the covers were completely removed 40 days after the start of the removal (Fig. 1.5).

With a photosynthetic photon flux (PPF) of 60  $\mu$ mol · m<sup>-2</sup> · s<sup>-1</sup> provided by cool-white fluorescent lamps, cultures were maintained at 22 °C under the 16 h/day

photoperiod, or at 28 °C under the 16 h/day photoperiod, at 28 °C under the 24 h/day photoperiod, at 34 °C under the 16 h/day photoperiod. Twenty rooted shoots were used per treatment, and the shoot length and the number of leaves of the acclimatized micropropagules were examined 50 days after the completion of acclimatization.

*Effect of PPF on acclimatization (Expt. 2)*

The method of the acclimatization was the same as that in Expt. 1. Cultures were maintained at 28 °C under the 24 h/day photoperiod with a PPF of 15 to 120  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Twenty-five rooted shoots were used per treatment, and the shoot length and the number of leaves of the acclimatized micropropagules were examined 50 days after the completion of acclimatization.

*Effect of planting time on acclimatization (Expt. 3)*

After the rooting treatment, the microshoots were planted in the pots directly or through the culture in the rooting medium. The periods of the culture were the following: 1) 10 days, during which half of the microshoots rooted. 2) 20 days, during which 80-90% of them rooted. 3) 40 days, during which almost all of them rooted.

After the dark incubation for initial 10 days after the rooting treatment to ensure rooting, cultures were maintained at 28 °C under the 24 h/day photoperiod with a PPF of 60  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for the potted microshoots or under the 16 h/day photoperiod for the microshoots in the rooting medium. Twenty-five microshoots were used per treatment, and the shoot length and the number of leaves of the acclimatized micropropagules were examined 50 days after the completion of acclimatization.

*Growth of acclimatized micropropagules after being transferred outside (Expt. 4)*

The acclimatized micropropagules were transplanted to the partly grazed pots 18 cm in diameter or to the outdoor nursery, and were covered with shade nets for a month. The medium in the pots was the same as that in the nursery. There were 98 plants in the pots and 82 plants in the nursery. During the growing season, each plant received a total of 12g of 8N-8P-8K fertilizer as a split application. The number of surviving plants, the plant height, and the trunk diameter at 5 cm above ground level were examined at the

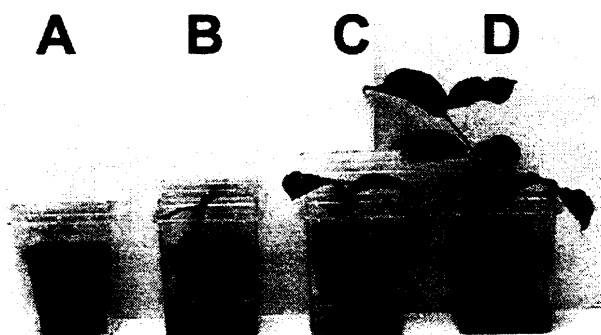


Fig. 1.5. The acclimatization method of Japanese persimmon 'Nishimurawase'. A: Transplanting in the pot, B-C: Acclimatization to low relative humidity, D: Completion of acclimatization.

end of the growing season.

## Results

### *Effect of temperature and photoperiod on acclimatization (Expt. 1)*

When the cultures were maintained at 28 °C under the 24 h/day photoperiod, 90 % of the micropropagules expanded new leaves after planting in the pots and were acclimatized. At 28 °C under the 16 h/day photoperiod, 75 % were acclimatized, and at 22 °C under the 16 h/day photoperiod, 70 % were acclimatized. The lowest percentage (65 %) of the micropropagules was acclimatized at 34 °C under the 16 h/day photoperiod. The mortality rate during and after acclimatization was lower when the culture was maintained at 28 °C irrespective of the photoperiod than those under the other culture conditions (Table 1.6). The acclimatized micropropagules cultured at 28

Table 1.6. Effects of temperature and photoperiod on the growth of micropropagules<sup>z</sup>.

| Conditions       |                 | Number of<br>micropropagules<br>examined | Shoot<br>length<br>(mm) | Number<br>of<br>leaves |
|------------------|-----------------|--|-------------------------|------------------------|
| Temperature (°C) | Photoperiod (h) |  |                         |                        |
| 22               | 16              | 6 <sup>y</sup>                           | 2.7 ± 0.6 <sup>x</sup>  | 3.8 ± 0.6 <sup>x</sup> |
| 28               | 16              | 14                                       | 8.0 ± 1.3               | 7.1 ± 0.9              |
| 28               | 24              | 17                                       | 10.9 ± 1.4              | 8.5 ± 0.7              |
| 34               | 16              | 4  | 2.8 ± 0.2               | 3.8 ± 0.5              |

<sup>z</sup> Data were taken 50 days after acclimatization. PPF was 60  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$

<sup>y</sup> Although 20 micropropagules were used for each treatment, some died during and after acclimatization.

<sup>x</sup> Mean  $\pm$  SE.

°C repeated growth of new shoots several centimeters long from their terminal or sub-terminal buds after a short pause, although the leaves cultured under the 24 h/day photoperiod were deeper green and the shoots were thicker than those under the 16 h/day photoperiod. The micropropagules cultured at 22 and 34 °C, however, stopped growing during or after acclimatization, and none of them survived 50 days after the completion of acclimatization.

### *Effect of PPF on acclimatization (Expt. 2)*

Most of the micropropagules cultured with the lower PPF grew weak and died before the start of acclimatization, whereas the micropropagules with the higher PPF died hardly during and after acclimatization, and grew vigorously (Table 1.7).

### *Effect of planting time on acclimatization (Expt. 3)*

Most of the microshoots planted directly in the pots did not root and died 20 days after planting in the pots (Table 1.8). Even though the rest rooted and were

acclimatized, none of them survived 40 days after the completion of acclimatization.

The rooting percentage 10 days after the rooting treatment was 52%, and those 20 and 40 days after the rooting treatment were 88% and 96%, respectively. When planted in the pots 10 days after the rooting treatment, almost all of the rooted shoots survived and grew well, and most of the unrooted shoots rooted after planting in the pots and expanded new leaves. When planted in the pots 20 or 40 days after the rooting treatment, almost all of the micropropagules also survived and grew well. The micropropagules that were cultured in the rooting medium before planting in the pots grew well after the completion of acclimatization, and the micropropagules cultured for 20 days in the rooting medium showed the most vigorous growth (Table 1.8).

Table 1.7. Effects of PPF on the growth of micropropagules<sup>z</sup>.

| PPF<br>( $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) | Number of<br>micropropagules<br>examined | Shoot<br>length<br>(mm) | Number<br>of<br>leaves |
|--|--|-------------------------|------------------------|
| 15   | 3 <sup>y</sup>                           | $1.7 \pm 0.3^x$         | $3.0 \pm 0.8^x$        |
| 30   | 15                                       | $6.2 \pm 1.0$           | $5.4 \pm 0.6$          |
| 60   | 24                                       | $11.7 \pm 0.9$          | $7.7 \pm 0.3$          |
| 120  | 25                                       | $13.0 \pm 0.9$          | $8.2 \pm 0.4$          |

<sup>z</sup> Data were taken 50 days after acclimatization. Cultures were maintained at 28 °C under the 24 h/day photoperiod.

<sup>y</sup> Although 25 micropropagules were used for each treatment, some died during and after acclimatization.

<sup>x</sup> Mean  $\pm$  SE.

Table 1.8. Effects of the culture period in the rooting medium on the growth of micropropagules<sup>z</sup>.

| Days of culture in the<br>rooting medium before<br>planting in the pots | Number of<br>micropropagules<br>examined | Shoot<br>length<br>(mm) | Number<br>of<br>leaves |
|---|--|-------------------------|------------------------|
| 0 <sup>y</sup>  | 1 <sup>x</sup>                           | $2.0 \pm 0^w$           | $3.0 \pm 0^w$          |
| 10  | 24                                       | $8.0 \pm 0.6$           | $7.1 \pm 0.4$          |
| 20  | 23                                       | $10.3 \pm 0.5$          | $8.9 \pm 0.3$          |
| 40  | 23                                       | $8.0 \pm 0.7$           | $8.0 \pm 0.5$          |

<sup>z</sup> Data were taken 50 days after acclimatization. Cultures were maintained at 28 °C under the 24 h/day photoperiod.

<sup>y</sup> Direct rooting in the pots containing vermiculite.

<sup>x</sup> Although 25 micropropagules were used for each treatment, some died during and after acclimatization.

<sup>w</sup> Mean  $\pm$  SE.

#### *Growth of acclimatized micropropagules after being transferred outside (Expt. 4)*

More than 90% of the plants in the pots and in the nursery survived after transplanting (Table 1.9). Most of the plants in the nursery grew continuously, and half of them became more than 1 m in height at the end of

Table 1.9. Effects of planting place on survival and growth of micropropagated plants<sup>z</sup>.

| Planting place    | Survival (%) | Plant height (cm)       | Trunk diameter <sup>y</sup> (mm) |
|-------------------|--------------|-------------------------|----------------------------------|
| Outdoor nursery   | 91           | 95.7 ± 3.6 <sup>x</sup> | 8.3 ± 0.3 <sup>x</sup>           |
| Partly glazed pot | 96           | 45.7 ± 1.7              | 7.2 ± 0.1                        |

<sup>z</sup> 82 micropropagated plants were planted in the outdoor nursery, and 98 in the partly glazed pots. They were planted in May, and data were taken at the end of the growing season.

<sup>y</sup> Measured at 5 cm above ground level

<sup>x</sup> Mean ± SE.

the growing season. However, the plants in the pots grew discontinuously, and the height of them was half of that of the plants in the nursery. Three plants in the pots bore flowers in late summer (Fig. 1.6). One plant had female flowers, another had male flowers, and the other had both flowers.

#### **Discussion**

The leaves from micropropagules of strawberry cultured in vitro were characterized by poorly-formed palisade cells and large air spaces, and did not fix sufficient carbon to maintain the micropropagules, but those leaves developing subsequent to transplanting to glasshouse conditions were characterized by well-developed palisade cells and smaller air spaces, and were photosynthetically capable (Fabbri et al., 1986; Grout and Millam, 1985). As a result, the vigorous growth of micropropagated strawberry plants after transplanting depended on development of new leaves. The in vitro leaves of Japanese persimmon also differed from those developing after transplanting morphologically. In the preliminary experiments, when the micropropagules were acclimatized before expanding new leaves, few of them expanded new leaves during acclimatization and almost all of them died. Hence, in this study, the small vessels for acclimatization were used so that each of the micropropagules expanding new leaves carried out acclimatization. Defoliation of in vitro leaves and GA<sub>3</sub> treatment were effective in inducing new leaves of the rootstocks for Japanese persimmon after transplanting to the pots (Kagami, 1995).

The conditions for in vitro culture of Japanese persimmon were generally established at 28 °C under the 16 h/day photoperiod or under the 24 h/day photoperiod,

and the same conditions were used during and after acclimatization (Cooper and Cohen, 1985; Fumuro et al., 1988). However, no report has examined the best conditions for in and ex vitro culture. In this study, the environmental conditions were examined in the first place, for the micropropagules were transplanted to the pots without carbon resource, shifted from heterotrophy to autotrophy, and were directly affected by the environmental conditions. The results showed that the largest number of the micropropagules survived under the same conditions as used in the in vitro culture and that the temperature was the crucial condition in the survival and the growth of the micropropagules. Hidaka and Kajiura (1989) also pointed out the importance of temperature for acclimatization of citrus micropropagules.

The shoot length of Japanese persimmon cultivars cultured in vitro under the 16 h/day photoperiod was similar to that under the 24 h/day photoperiod, except 'Nagara' (Murayama et al., 1989; Tetsumura et al., 1991). In this study, however, the micropropagules under the 24 h/day photoperiod after planting in the pots grew more vigorously than those under the 16 h/day photoperiod, which indicated the light condition during and after acclimatization was also important for the growth of the micropropagules. On the other hand, the



Fig. 1.6. Micropropagated plants of Japanese persimmon 'Nishimurawase' bore both male and female flowers. A: The whole plants, bar indicates 50 cm, B: Arrow indicates a female flower, C: Arrows indicate male flowers.



relation of the micropropagules' growth to CO<sub>2</sub> concentration must be evident, because the air surrounding micropropagules changes after the removal of the vessels' covers. Incidentally, the quantity of light energy, not the photoperiod, probably affected the growth of micropropagules during and after acclimatization, because the differences in the survival percentage and the shoot length in Exp. 1 and 2 were similar to the difference in the quantity of light energy rather than in the photoperiod. Hence, the enhancement of light energy during and after acclimatization should produce better survival and growth, although the optimum quantity of light energy was not definite in this study. Further investigation on the relation of light energy and CO<sub>2</sub> concentration to survival and growth of micropropagules is needed.

The microcuttings of M.26 apple rootstocks survived and grew better after transplanting to propagation trays containing vermiculite when they were cultured in the rooting medium for a shorter period, and plenty of micropropagated plants were obtained by substituting the in vitro rooting stage with rooting in mist (Simmonds, 1983). The in vitro shoots of *Camellia japonica* cv. Alba Plena transplanted to glasshouse 12 days after the rooting treatment, when they did not root yet, survived after acclimatization better than those transplanted immediately or four weeks after the rooting treatment (Vieitez et al., 1989). Microcuttings of *Zelkova serrata* transplanted from 6-8 weeks rooting culture to pots developed roots poorly and survived insufficiently, whereas 90% of those transplanted from 10-12 weeks rooting culture to pots survived (Tomita, 1992). In this study, 'Nishimurawase' microcuttings were successfully acclimatized, when they were cultured in the rooting medium for a certain period of time, especially for 20 days.

As for acclimatization of *Euphorbia fulgens* microcuttings, survival rate increased with microcutting length (Zhang and Stoltz, 1989). Fumuro et al. (1988) pointed out the importance of using of vigorous microcuttings of 'Nishimurawase' Japanese persimmon for acclimatization. Hence, the microcuttings used in this study were more than 15 mm in length and had more than three leaves.

The field performance of micropropagated plants was considerably well in the first growing season. Therefore, the micropropagated plants establishing outdoors could be handled at the end of the growing season as easily as the conventionally propagated plants. Japanese persimmon is particularly prone to transplantation shock (Izaki et al., 1958; Kitagawa and Glucina, 1984), although the micropropagated plants of Japanese persimmon had well-developed root systems in comparison with the plants grafted on free stock seedlings (Tao et al., 1994). There is a need of further investigation to compare the growth of micropropagated trees with that of trees grafted on seedlings

after orchard establishment.

The rejuvenation and the precocity of micropropagated fruit trees are important concerns. In this study, the micropropagated plants planted in the pots bore flowers, which indicated that the true rejuvenation did not occur to the micropropagated plants, although the plants planted in the outdoor nursery did not. Tao et al. (1994) also concluded that the partial rejuvenation or reinvigoration, but not true rejuvenation, occurred to the micropropagated trees of 'Jiro' Japanese persimmon.

### **Summary**

The factors influencing the acclimatization of micropropagules of 'Nishimurawase' Japanese persimmon derived from shoot tips were studied, and their growth after being transferred outside was evaluated. During acclimatization, the optimum micropropagules survival was at 28 °C, but most died at 22 or 34 °C. Micropropagules grew better under the 24 h/day photoperiod than under the 16 h/day photoperiod. Enhanced PPF stimulated the growth of the micropropagules and raised their survival rate. When microcuttings were transplanted in the pots immediately after the rooting treatment, none survived. On the other hand, when transplanted after root induction in the rooting medium, almost all of them survived. The highest growth rate was obtained with those microcuttings cultured for 20 days in the rooting medium. Although micropropagules grew vigorously when transferred outside, they grew better when transplanted in the outdoor nursery than in the partly grazed pots. The true rejuvenation did not occur in the shoot tip culture of Japanese persimmon, because some of the micropropagated plants in the pots bore both male and female flowers four months after being transferred outside.

## **Chapter 2.**

### **Production of Own-rooted Trees by Cutting Propagation**

#### **Section 1. Hardwood Cuttings**

##### **Introduction**

The results from *in vitro* experiments (Chapter1-1-II) confirmed that the shoots differentiated from the root pieces of Japanese persimmon cultivars rooted better than those derived from the shoot tips. Furthermore, successful rooting of the softwood cuttings of a potentially dwarfing rootstock for Japanese persimmon was achieved by using the shoots differentiated from *in vivo* roots, i.e., root suckers (Tetsumura et al., 2000a). In a preliminary trial with rooting of hardwood cuttings collected from 'Nishimurawase' Japanese persimmon root suckers, they were proved to root, especially when mounded at their base until early summer in a similar way as stool-layering, but the optimum planting time of cuttings and the best method of rooting treatment were still unclear (Tetsumura et al. 1998). In this section, the effects of planting time, methods of rooting treatment, mounding, and cultivar on rooting of hardwood cuttings collected from root suckers of Japanese persimmon cultivars, whose mother trees had been micropropagated, were examined as compared with those of hardwood cuttings collected from the shoots of micropropagated or conventionally grafted trees.

##### **Materials and Methods**

###### *Cutting source*

In January 1991, 'Nishimurawase' Japanese persimmon trees raised by micropropagation and conventional grafting were planted at the orchard of the Experimental Farm of Kyoto University, as described in Chapter 3-1-II. In April 1995, the micropropagated trees, including the root shanks, were cut down just above the ground level. Then, the surface soil about 1 m<sup>2</sup> around the stump and 30 cm in depth was removed, and part of the root more than 1.5 cm in diameter was exposed to stimulate differentiation of root suckers. Micropropagated 'Jiro' trees were also planted in March 1993 and their tops were cut off in March 1997. Root suckers were induced in the same way as 'Nishimurawase'. Conventionally grafted 'Jiro' trees (over 65 years) were also used for the cutting source from shoots.

Five types of source shoots were prepared for the hardwood cuttings used in this study. Some root suckers had been mounded (etiolated) with rice husks at their base since late-June and the others remained exposed (unmounded). The basal or middle part

of the root suckers was collected for cuttings. The cuttings from micropropagated and conventionally grafted trees were collected from the basal part of vigorous non-bearing one-year-old shoots. All the materials were harvested in mid-December and stored at 3 °C in a refrigerator until the rooting treatment. All the cuttings were cut into 25-cm long pieces just before the rooting treatment and the base was over 7 mm in diameter.

Some etiolated root suckers had already rooted in the mounds. After being separated from the mother plants, they were subjected to the same rooting treatments and planted in the same bottom-heated medium as used in Exp. 1. Their survival (establishment) was estimated at the end of the growing season.

#### *Effect of rooting treatments (Expt. 1)*

The experiments were conducted in 1999 using 10 cuttings per type for each treatment. On March 11, in one group, the base was soaked for 24 hr in an aqueous solution of 25 ppm IBA, and then for 30 min in a solution of 0.5 % benomyl. In another group, the base was soaked in water for 24 hr, and then dipped for 5 sec in a 3000 ppm IBA solution with 50% aqueous ethanol solution followed by soaking in benomyl solution, or soaked only in the benomyl solution (control). After the rooting treatments, the cuttings were planted upright in a mixture of Kanuma soil (2 to 3 mm in diameter) and peat moss (1:1, v:v) in 20-liter plastic netted baskets 17 cm in depth, 20 cuttings per basket. The baskets with cuttings were placed under a shade net with vaporized aluminum in a propagation frame covered with plastic film. The propagation frame was intermittently misted, and ventilated with fans, which turned on when the temperature exceeded 10 °C. Bottom heat was supplied at 28 °C by thermostatically-controlled low voltage heating wires, which were laid under the baskets. The baskets were transferred outdoors 60 days after the planting. Failed cuttings were checked at intervals and discarded. At the end of the growing season, the surviving cuttings were counted as established cuttings because all of them rooted well, and the length and number of their annual shoots was recorded.

#### *Effect of planting time (Expt. 2)*

The experiments were conducted in 1998 and 1999 using 10 cuttings per type. In late-January, late-February, and late-March, the base was soaked in a 25 ppm IBA solution for 24 hr, and then in a 0.5 % benomyl solution for 30 min. After this rooting treatment, the cuttings were planted in the same rooting medium and under the same environmental conditions as in Exp. 1. The rooting and establishment percentages were examined, and the length and number of annual shoots were recorded at the end of the growing season.

#### *Statistical analysis*

The  $5 \times 3 \times 2$  factorial with cutting source, method of rooting treatment (Exp. 1) or planting time (Exp. 2), and cultivar as main effects was analyzed by ANOVA. All percentage data were subjected to arcsin transformation before subjected to ANOVA. In Exp. 2, the years (1998 and 1999) served as the replication only in the experimental design.

#### *Histological observations*

For observations of the root primordia formed and developed before the rooting treatment, the basal part of the cutting derived from 'Jiro' root suckers was fixed in FAA (70% ethanol: formalin: acetic acid, 90:5:5, v/v/v), treated with the softening agents according to Nakamura (1985), dehydrated in n-butyl alcohol series, embedded in paraffin, and cut transversely into 10  $\mu\text{m}$  sections. The sections were stained with 0.05 % toluidine blue O and observed under a light microscope.

### **Results**

#### *Cutting sources*

In late-April, many root suckers sprouted from the exposed part of the root. Every year, each tree of both cultivars produced 20 to 30 root suckers that grew well enough to be used for hardwood cuttings.

When harvested, half of etiolated 'Jiro' suckers had already rooted in the mounds and one-third of etiolated 'Nishimurawase' suckers had rooted. The former had 2.3 roots per rooted sucker and the latter had 2.4 roots. Irrespective of the IBA treatments, 80 % of the rooted suckers that were planted in the rooting medium formed new roots, and 70 % were established at the end of the growing season.

#### *Effect of rooting treatments (Expt. 1)*

As compared with the control, the method of IBA treatment for rooting had no effect on the rooting percentage (Table. 2.1). There was no significant difference in the rooting percentage between 'Jiro' and 'Nishimurawase' cuttings. However, the cutting source had a significant effect on the rooting. The highest rooting (52 %) was achieved by the cuttings from basal part of mounded root suckers. Only 3 % of the cuttings from the middle part of root suckers rooted, while 27 % of those from basal part of unmounded root suckers rooted. The cuttings from shoots of micropropagated trees or of conventionally grafted trees did not root at all. Significant interaction was observed between the cutting source and the rooting treatment or cultivar. At the end of the growing season, almost all of the rooted cuttings survived, and the number of shoots and the total shoot length of the established cuttings were 2.4 and 41 cm, respectively. These values were not significantly affected by either the cutting source, or the method

of rooting treatment, or the cultivar (data not shown).

Table 2.1. Effects of cutting source, method of rooting treatment, and cultivar on the rooting of Japanese persimmon hardwood cuttings (Expt. 1).

| Factor                                 | Rooting (%)      |
|--|------------------|
| <i>Cutting source</i>                  |                  |
| Basal part of mounded root suckers     | 52               |
| Basal part of unmounded root suckers   | 27               |
| Middle part of root suckers            | 3                |
| Shoots of micropropagated trees        | 0                |
| Shoots of conventionally grafted trees | 0                |
| <i>Method of rooting treatment</i>     |                  |
| Control                                | 17               |
| IBA 25 ppm for 24 hr                   | 16               |
| IBA 3000 ppm for 5 sec                 | 16               |
| <i>Cultivar</i>                        |                  |
| Jiro                                   | 17               |
| Nishimurawase                          | 16               |
| <i>Significance</i>                    |                  |
| Cutting source (CS)                    | *** <sup>z</sup> |
| Method of rooting treatment (RT)       | ns               |
| Cultivar (CV)                          | ns               |
| CS × RT                                | *                |
| RT × CV                                | ns               |
| CV × CS                                | *                |

<sup>z</sup> ns, \*, \*\*\* : nonsignificant or significant at  $P < 0.05$  or  $0.005$ , respectively.

#### *Effect of planting time (Expt. 2)*

The planting time had no effect on the rooting percentage (Table. 2.2). The rooting percentages were similar to those in Exp. 1 for all cutting sources and cultivars. Only the cutting source showed a significant effect, and the cultivar showed a significant interaction with the cutting source. The planting time did not affect either the number of shoots or the total shoot length of the established cuttings (data not shown). The early spring in 1998 was warmer than that in 1999, so the cuttings in 1998 sprouted 10 days earlier than those in 1999. However, there was no apparent difference in rooting percentage between them (data not shown).

Table 2.2. Effects of cutting source, planting time, and cultivar on the rooting of Japanese persimmon hardwood cuttings (Expt. 2).

| Factor                                 | Rooting (%)      |
|--|------------------|
| <i>Cutting source</i>                  |                  |
| Basal part of mounded root suckers     | 49               |
| Basal part of unmounded root suckers   | 24               |
| Middle part of root suckers            | 2                |
| Shoots of micropropagated trees        | 0                |
| Shoots of conventionally grafted trees | 0                |
| <i>Planting time</i>                   |                  |
| Late-January                           | 18               |
| Late-February                          | 14               |
| Late-March                             | 14               |
| <i>Cultivar</i>                        |                  |
| Jiro                                   | 13               |
| Nishimurawase                          | 17               |
| <i>Significance</i>                    |                  |
| Cutting source (CS)                    | *** <sup>z</sup> |
| Planting time (PT)                     | ns               |
| Cultivar (CV)                          | ns               |
| CS × PT                                | ns               |
| PT × CV                                | ns               |
| CV × CS                                | *                |
| CS × PT × CV                           | ns               |

<sup>z</sup> ns, \*, \*\*\* : nonsignificant or significant at  $P < 0.05$  or  $0.005$ , respectively.

### *Histological observations*

The adventitious root primordia were induced at various differentiation stages in the basal part of cuttings from basal part of mounded root suckers (Fig. 2.1A-C). These primordia originated in phloem in association with rays, and were found under lenticels. The basal part of the cuttings from basal part of mounded root suckers had a thick-layered periderm with well-developed phellogen and phellem, and had few sclerenchymatous cells (Fig. 2.1D). Root primordia could not be found in the basal part of the cuttings from basal part of unmounded root suckers, which had a discontinuous sclerenchymatous ring in cortex, and a several-layered and lignified periderm (Fig.

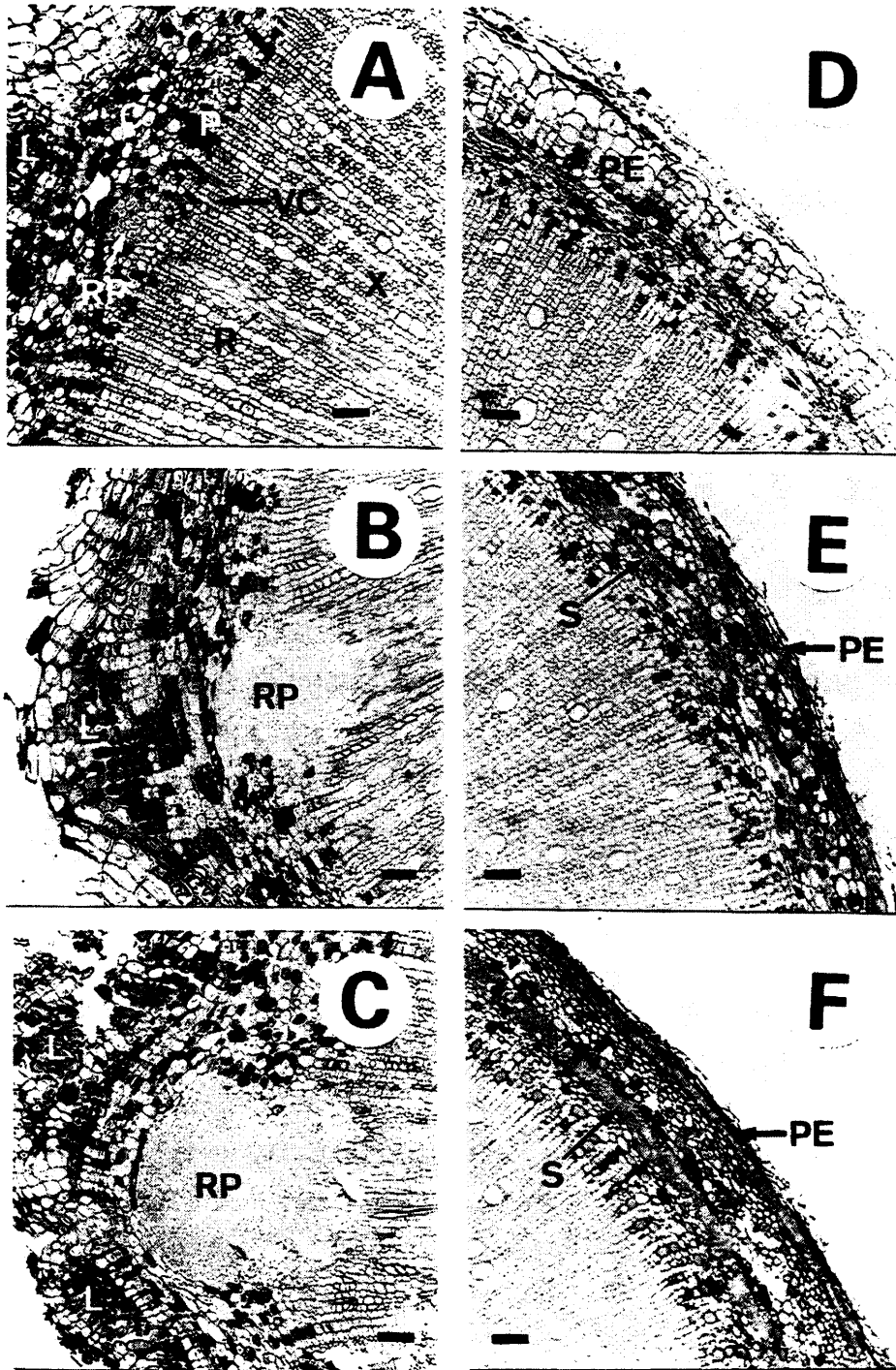


Fig. 2.1. Transverse sections of basal part of hardwood cuttings derived from basal part of mounded root suckers (A, B, C, and D), basal part of an unmounded root sucker (E), and middle part of a root sucker (F) of 'Jiro' Japanese persimmon before the rooting treatment. (A) An initial adventitious root primordia in phloem in association with rays. (B) A well-developed adventitious root primordia under a lenticel. (C) An adventitious root primordia pushing out tissues. (D) A thick-layered periderm with well-developed phellogen and phellem. (E) A discontinuous sclerenchymatous ring in cortex and a several-layered and lignified periderm. (F) A continuous and thick sclerenchymatous ring and a well-lignified periderm. Abbreviations: cortex (C), lenticel (L), periderm (PE), phloem (P), ray (R), root primordium (RP), sclerenchyma (S), vascular cambium (VC), xylem (X). All scale bars = 0.1 mm.



2.1E). Root primordia could not be found in the basal part of the cuttings from middle part of root suckers either, which had only a continuous and thick sclerenchymatous ring and a well-lignified periderm (Fig. 2.1F).

## **Discussion**

Although some etiolated root suckers rooted in the mounds and were easily established in the rooting medium without IBA treatment, more improvement in the rooting percentage and the root number will be needed to supply etiolated suckers as stool shoots. As for apple rootstocks that have been commercially propagated by stool-layering, the stool shoots had 13.4 to 58.6 roots per shoot (Doud and Carlson, 1977), and 70 to 100 % rooting is recommended (Macdonald, 1986). Girdling was reported to improve rooting of chestnut, a difficult-to-root fruit species (Biricolti et al., 1994), and it may be applied to Japanese persimmon.

As observed in the preliminary trial (Tetsumura et al. 1998), IBA treatment did not improve rooting of the cuttings. Generally, exogenous IBA treatment improved rooting of hardwood cuttings (Erez and Yablowitz, 1981; Howard, 1978; Howard and Nahlawi, 1969), even of the unrooted stool shoots of M.27 apple rootstock (Pontikis et al., 1979). The greatest effect of exogenous auxin on adventitious root formation was expected to occur during early primordium development (Haissig, 1970; Norcini et al. 1985). Histological observations in this study revealed that the cuttings from mounded basal root suckers had formed adventitious root primordia before IBA treatments. These primordia developed probably into roots irrespective of IBA treatment. This seemed to make IBA treatment ineffective for rooting of the cuttings from mounded basal root suckers.

However, the cuttings from basal part of unmounded root suckers, in which any primordia could not be found, also rooted to some degree irrespective of IBA treatment. Preliminary experiments revealed that the roots of cuttings from basal part of mounded root suckers were visible 20 days after planting while those from basal part of unmounded root suckers were visible 40 days after planting. Hence, the cuttings from basal part of unmounded root suckers were assumed to form root primordia after planting. A possible reason for the ineffectiveness of IBA treatment might be the degradation of IBA before it could exert an effect. The rooting of peach and apple hardwood cuttings treated with IBA occurred from 10 to 20 days after planting (Gemma et al., 1983; Howard, 1980). Further observations of the root primordia formation and the change of IBA in the cuttings from basal part of unmounded root suckers during planting are needed to examine this possibility. Also, the concentration of IBA may

have been inadequate.

The planting time of the cuttings was not an important factor influencing their rooting, as observed in the preliminary trial (Tetsumura et al. 1998). The rooting ability of the hardwood cuttings of apple and plum was reported to be low in midwinter, but to rise toward spring (Howard, 1965; Howard, 1980; Howard and Nahlawi, 1969). Bassuk and Howard (1981) found that the activities of rooting cofactors in M.26 apple hardwood cuttings were closely correlated with the seasonal rooting response of cuttings, and Gesto et al. (1981) showed that the inhibitors of rooting of chestnut cuttings decreased with the length of cold storage period. However, it seemed unlikely that such promotive or inhibitory substances existed in the hardwood cuttings of Japanese persimmon.

Mounding brought about histological changes in the root suckers. Even though not mounded, the basal part of root suckers was somewhat etiolated due to shading with the crowding of leaves from June to November. The basal part of cuttings from middle part of root suckers had well-developed sclerenchyma, while those from basal part of mounded root suckers had poorly-developed sclerenchyma and those from basal part of unmounded root suckers had moderately-developed sclerenchyma. Many investigators have proposed that the development of sclerenchyma reduced the rooting potential of cuttings (Biricolti et al., 1994; Doud and Carlson, 1977; Maynard and Bassuk, 1996). The development of sclerenchyma might also affect the formation of root primordia in the cuttings of Japanese persimmon after planting, because the cuttings from basal part of unmounded root suckers rooted to some degree but those from middle part of root suckers scarcely rooted. Incidentally, the lenticels, paths of water and air through the periderm of root suckers in the mound, might help the formation of root primordia, as has been reported with some trees (Machida and Fujii, 1969).

In addition to the favorable effect of mounding, the fact that the cuttings were taken from root suckers was also favorable for rooting, because they are physiologically juvenile (Del Tredici, 1995). Higdon and Westwood (1963) pointed out that juvenile 'Old Home' pear cuttings, which rooted better than adult cuttings, were obtained from adventitious shoots forced from scion roots of 'Old Home'. Successful propagation by softwood cuttings of a potentially dwarfing rootstock for Japanese persimmon by using the shoots from root suckers was also reported (Tetsumura et al., 2000a).

The use of mounded basal root suckers of hardwood cuttings of Japanese persimmon cultivars is a promising propagation method. Moreover, a subsidiary trial with another Japanese persimmon cv., Hiratanenashi, resulted in successful propagation in the same manner. Hence, this vegetative propagation method should be applicable to

many other persimmon cultivars.

### Summary

The factors influencing rooting of hardwood cuttings from two cvs., Jiro and Nishimurawase, of Japanese persimmon were studied. After the micropropagated trees were cut down, the root suckers sprouted from the roots, and a quarter of the cuttings collected from their basal part rooted. Half of the cuttings from the root suckers whose basal part had been etiolated by mounding rooted. Some of the mounded root suckers had already rooted in the mounds, and were easily established in the bottom-heated medium after being separated from the mother plants. The cuttings from the one-year-old shoots of micropropagated or conventionally grafted trees did not root. There was no significant difference in the rooting percentage between 'Jiro' and 'Nishimurawase' cuttings. In comparison with the control, neither keeping the cutting base in 25 ppm IBA for 24 hr nor dipping in 3000 ppm IBA for 5 sec improved the rooting percentage. The planting time, from late-January to late-March, had no effect on the rooting percentage. Almost all of the rooted cuttings grew well and were established in the rooting medium. Before the rooting treatment, adventitious root primordia at various differentiation stages were found in the basal part of the cuttings from the basal part of mounded root suckers, in which well-developed periderm and few sclerenchymatous cells were observed. Root primordia were not observed in the basal part of cuttings from the basal part of unmounded root suckers or from the middle part of root suckers. The former had a discontinuous sclerenchymatous ring and rooted to some degree, although the latter had a well-developed continuous sclerenchymatous ring and scarcely rooted.

## **Section 2. Softwood Cuttings**

### **Introduction**

Although there has been no report of hardwood cutting propagation of Japanese persimmon cultivars, there have been a few reports on the successful propagation with softwood cuttings (Machida and Fujii, 1969; Murata et al., 1983). In all of their trials, however, the etiolation of stock plants and the blanching of cutting bases were required to obtain good rooting. These operations require a lot of time. Therefore, these propagation methods have never been used commercially.

The previous section showed the successful propagation with hardwood cuttings of two Japanese persimmon cultivars by using their root suckers. Furthermore, the successful rooting of the softwood cuttings of a potentially dwarfing rootstock for Japanese persimmon was achieved by using its root suckers (Tetsumura et al., 2000a). When planted in late June or late July, almost all of the single-node stem (leaf-bud) cuttings rooted. Moreover, without any cumbersome operation, many suckers were produced annually, and the leaf-bud cuttings supplied hundreds of materials for mass propagation. Therefore, the conclusion was that the propagation method would be promising for commercial use. In this section, to apply the propagation method to Japanese persimmon cultivars, the effects of cutting length, methods of rooting treatment, planting time, and cultivar on rooting of softwood cuttings taken from root suckers, whose mother trees had been micropropagated, were examined in comparison with those of softwood cuttings taken from the shoots of micropropagated trees and grafted trees. Furthermore, the histological changes in the cutting bases in relation to rooting and survival of the cuttings after planting were observed.

### **Materials and Methods**

#### *Effect of cutting source and cutting length (Expt. 1)*

In late June, softwood cuttings were taken from the root suckers of micropropagated trees and the over 50-cm-long and non-bearing shoots of micropropagated or grafted trees of 'Nishimurawase'. These stock plants were prepared in the same manner as described in Chapter 2-1. After the uppermost soft part of the root suckers and the shoots were discarded, three types of cuttings were prepared from the random position of the root suckers and the shoots: 1) 25-cm long cuttings with 5-7 buds and two halved terminal leaves; 2) 10-cm long cuttings with 3-4 buds and two halved terminal leaves; 3) 3-4 cm long cuttings with one bud and one leaf (leaf-bud cuttings). The bases of 10 cuttings of each type were dipped for 5 sec in 3000 ppm IBA

in 50% aqueous ethanol solution. After the rooting treatment, the cuttings were planted upright in a mixture of Kanuma soil (2 to 3 mm in diameter) and peat moss (1:1, v:v) in 20-liter plastic netted baskets 17 cm in depth, 20 cuttings per basket. The baskets with cuttings were placed under a shade net with vaporized aluminum in a propagation frame covered with plastic film, which provided 24% irradiance (photosynthetically active radiation) of the outside. The propagation frame was intermittently misted by using a screen-balance switch, which turned the mist sprayer on when the netted screen became dry, and was ventilated with fans, which turned on when the temperature exceeded 35 °C. The rooting percentage and the number of roots per rooted cutting were recorded 60 days after planting.

*Effect of cultivar, planting time and method of rooting treatment (Expt. 2)*

In late June, late July, and late August, leaf-bud cuttings were collected from the root suckers of micropropagated trees of 'Nishimurawase' and 'Jiro'. The bases of 10 cuttings of each cultivar were dipped for 5 sec in a 3000 ppm IBA in 50% aqueous ethanol solution. The bases of other 10 cuttings were soaked for 24 hr in an aqueous solution of 25 ppm IBA, while those of 10 cuttings (control) were soaked for 24 hr in water. After this rooting treatment, the cuttings were planted in the same rooting medium and under the same environmental conditions as in Exp. 1. The results were recorded 60 days after planting.

*Statistical analysis*

The 3 × 3 factorial with cutting source and cutting length as main effects was analyzed by ANOVA in Exp. 1. The 2 × 3 × 3 factorial with cultivar, planting time, and method of rooting treatment was analyzed by ANOVA in Exp. 2. All percentage data were subjected to arcsin transformation before subjected to ANOVA. In both experiments, the years (1997, 1998, and 1999) served as the block (replication) only in the randomized complete-block experimental designs.

*Changes with time in survival, rooting, and histology (Expt. 3)*

On June 16, 1999, the leaf-bud and the 25-cm cuttings, both from root suckers of micropropagated 'Jiro' trees, and the leaf-bud cuttings from the shoots of grafted trees of 'Jiro' were subjected to the same rooting treatment, and were planted in the same rooting medium under the same environmental conditions as in Exp. 1. On 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 days after planting, 10 cuttings of each type were dug up and examined for their survival and rooting. Then, their basal parts were excised, fixed in FAA (70% ethanol: formalin: acetic acid, 90:5:5, v/v/v), treated with the softening agents according to Nakamura (1985), dehydrated in n-butyl alcohol series, embedded in paraffin, and cut transversely into 10-14 μm sections. The sections were stained with

0.05 % toluidine blue O and observed under a light microscope.

## Results

### *Effect of cutting source and cutting length (Expt. 1)*

The cuttings from the root suckers of micropropagated trees rooted best, and the cuttings from the shoots of micropropagated trees rooted better than those from grafted trees (Table 2.3). Except for the cuttings from grafted trees, the shorter the cuttings, the higher the rooting percentage. The rooted cuttings with higher rooting percentage had more roots (data not shown): there was a linear relationship between the rooting percentage and the number of roots per rooted cutting [ $y (\%) = 11.43 + 4.77x (\text{number})$ ,  $r = 0.74$ ].

**Table 2.3. Effects of cutting source and cutting length on the rooting percentage of the softwood cuttings of 'Nishimurawase' Japanese persimmon, which were treated with 3000 ppm IBA solution for 5 sec (Expt. 1).**

| Cutting source                        | Cutting length (cm) | Rooting (%)      |
|---------------------------------------|---------------------|------------------|
| Root suckers of micropropagated trees | 25                  | 30               |
|                                       | 10                  | 43               |
|                                       | 3-4 <sup>z</sup>    | 70               |
| Shoots of micropropagated trees       | 25                  | 13               |
|                                       | 10                  | 17               |
|                                       | 3-4 <sup>z</sup>    | 37               |
| Shoots of grafted trees               | 25                  | 10               |
|                                       | 10                  | 7                |
|                                       | 3-4 <sup>z</sup>    | 10               |
| <i>Significance</i>                   |                     |                  |
| Cutting source (CS)                   |                     | *** <sup>y</sup> |
| Cutting length (CL)                   |                     | *                |
| CS × CL                               |                     | ns               |

<sup>z</sup> Leaf-bud cuttings.

<sup>y</sup> ns, \*, \*\*\* : nonsignificant or significant at  $P < 0.05$  or 0.005, respectively.

### *Effect of cultivar, planting time and method of rooting treatment (Expt. 2)*

The planting time and the method of rooting treatment had significant effects on the rooting percentages, and there was a significant interaction between them (Table 2.4). When the cuttings were planted in late-June, the IBA treatment was a requisite to obtain

Table 2.4. Effects of cultivar, planting time, and method of rooting treatment on the rooting percentage of leaf-bud cuttings from root suckers of micropropagated Japanese persimmon (Expt. 2).

| Cultivar                         | Planting time | Rooting (%)                 |                         |                           |
|----------------------------------|---------------|-----------------------------|-------------------------|---------------------------|
|                                  |               | Method of rooting treatment |                         |                           |
|                                  |               | Control                     | IBA 25 ppm<br>for 24 hr | IBA 3000 ppm<br>for 5 sec |
| Nishimurawase                    | Late-June     | 13                          | 73                      | 80                        |
|                                  | Late-July     | 0                           | 20                      | 37                        |
|                                  | Late-August   | 0                           | 10                      | 17                        |
| Jiro                             | Late-June     | 10                          | 77                      | 70                        |
|                                  | Late-July     | 0                           | 3                       | 30                        |
|                                  | Late-August   | 0                           | 7                       | 17                        |
| <i>Significance</i>              |               |                             |                         |                           |
| Cultivar (CV)                    |               | ns <sup>2</sup>             |                         |                           |
| Planting time (PT)               |               | ***                         |                         |                           |
| Method of rooting treatment (RT) |               | ***                         |                         |                           |
| CV × PT                          |               | ns                          |                         |                           |
| PT × RT                          |               | **                          |                         |                           |
| RT × CV                          |               | ns                          |                         |                           |
| CV × PT × RT                     |               | **                          |                         |                           |

<sup>2</sup> ns, \*\*, \*\*\* : nonsignificant or significant at  $P < 0.01$  or  $0.005$ , respectively.

more than 70% rooting, although there was no distinct difference in rooting percentages between the treatments with 25 ppm IBA for 24 hr and 3000 ppm IBA for 5 sec. When the cuttings were planted in late July or late August, however, the rooting percentages were much lower than those in late-June and the cuttings treated with IBA 3000 ppm rooted better than those with 25 ppm IBA. The control cuttings scarcely rooted even when planted in late June, and none of them rooted when planted in late July or late August. There was no significant difference in rooting percentage between the cultivars.

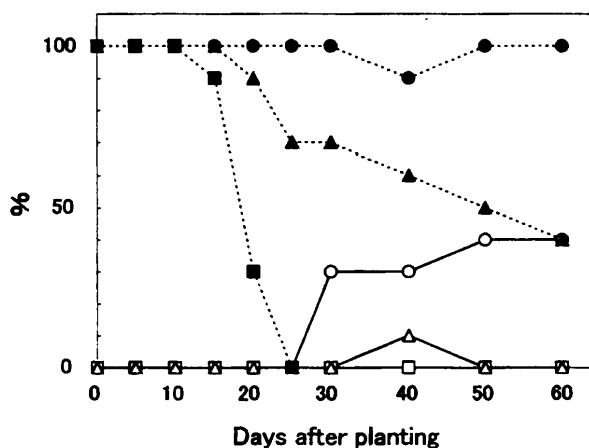


Fig. 2.2. Changes with time in rooting percentage (open symbols) and survival rate (solid symbols) of the softwood cuttings of 'Jiro' Japanese persimmon, which were treated with 3000 ppm IBA solution for 5 sec (Expt. 3).

○, ●: Leaf-bud cuttings from root suckers of micropropagated trees.

□, ■: Twenty-five-cm cuttings from root suckers of micropropagated trees.

△, ▲: Leaf-bud cuttings from shoots of grafted trees.

There was the same linear relationship between the rooting percentage and the number of roots per rooted cutting as in Exp. 1 [ $y (\%) = 15.44 + 8.18x (\text{number})$ ,  $r = 0.78$ ].

*Changes with time in survival, rooting, and histology (Expt. 3)*

Almost all of the leaf-bud cuttings from the root suckers of micropropagated trees survived 60 days after planting, although none of the 25-cm cuttings survived 25 days after planting (Fig. 2.2). The leaf-bud cuttings from the shoots of grafted trees died gradually, but 40% of them survived 60 days after planting. Rooting of the leaf-bud cutting taken from the root suckers was first observed 30 days after planting, although the final rooting percentage was lower than that in Exp. 2. The leaf-bud cuttings taken from the shoots hardly rooted and the 25-cm cuttings did not root at all.

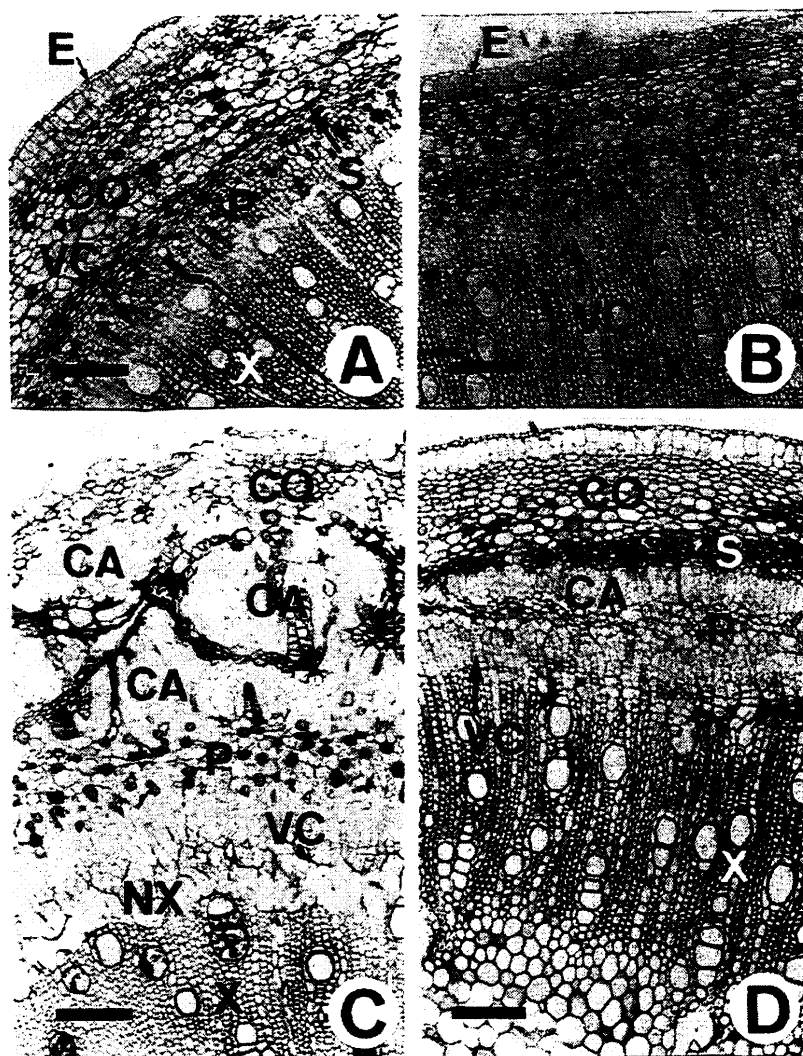


Fig. 2.3. Transverse sections of basal parts of the softwood cuttings of 'Jiro' Japanese persimmon, which were treated with 3000 ppm IBA solution for 5 sec. (A) 0 day after planting of leaf-bud cutting from the root sucker of micropropagated tree, showing few sclerenchymatous cells inside cortex. (B) 0 day after planting of leaf-bud cutting from the shoot of grafted tree, showing a continuous sclerenchymatous ring inside cortex. (C) 15 days after planting of leaf-bud cutting from the root sucker, showing callus developing extensively in the phloem and cortex and new xylem forming inside vascular cambium. (D) 15 day after planting of leaf-bud cuttings from the shoot, showing callus between a thick sclerenchymatous ring and phloem. Abbreviations: callus (CA), cortex (CO), epidermis (E), newly formed xylem (NX), phloem (P), sclerenchyma (S), vascular cambium (VC), xylem (X). All scale bars = 0.2 mm.



At the planting time, there was no root primordium in the cuttings and no apparent difference in histology between the cuttings from the root suckers and from the shoots, except for more distinct development of sclerenchyma in the latter (Fig. 2.3A-B). Vascular cambium of the leaf-bud cuttings from the root suckers began cell division soon after planting and formed new xylem. At the basal part of the cuttings, callus tissue formed in phloem and cortex at random, developed extensively, and broke through the cortex and epidermis (Fig. 2.3C). The basal tissues of the leaf-bud cuttings taken from the shoots were initially unchanged, but some of them began cell division 15 days after planting (Fig. 2.3D). Tissues of the 25-cm cuttings did not grow at all and collapsed gradually after planting.

Adventitious root initial cells, which could be readily distinguishable from callus cells because they differentiated in vascular cambium and in non-basal part of the

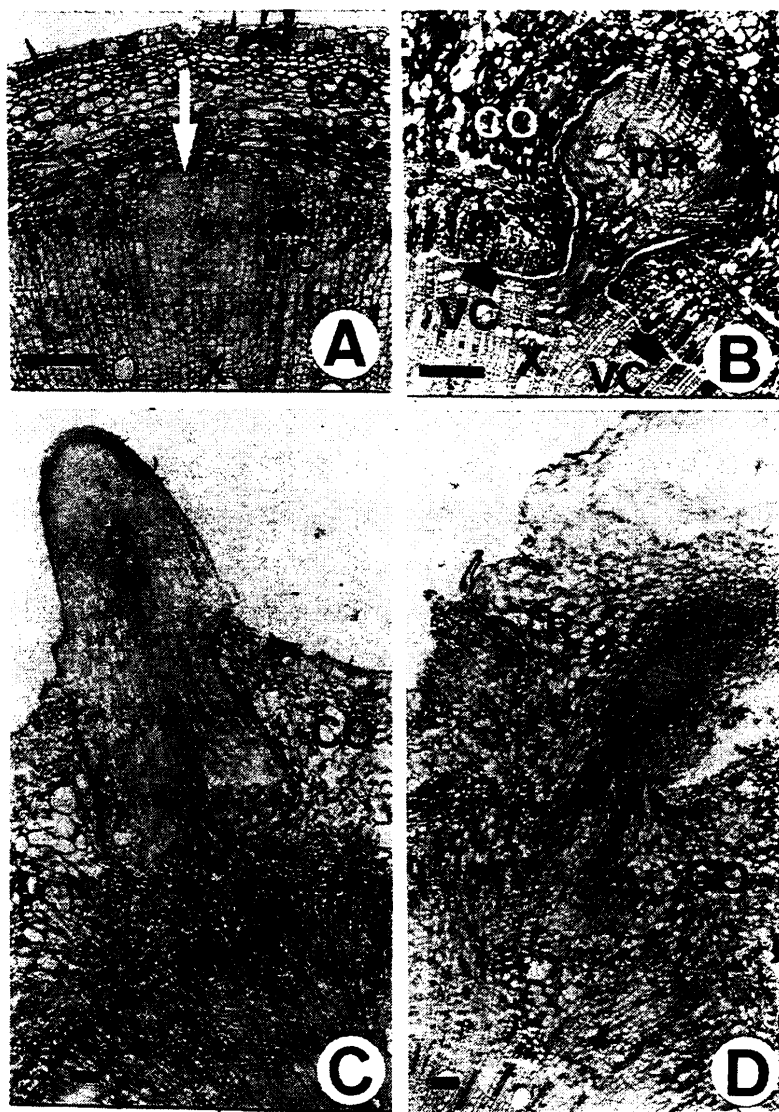


Fig. 2.4. Transverse sections of basal parts of leaf-bud cuttings, which were derived from the root suckers of 'Jiro' Japanese persimmon and treated with 3000 ppm IBA solution for 5 sec. (A) 20 days after planting, showing differentiation of distinct adventitious root initial cells in the vascular cambium (a white arrow). (B) 25 days after planting, showing development of a root primordium penetrating into phloem and cortex. (C) 30 days after planting, showing emergence of a root. (D) 40 days after planting, showing completion of vascular connections between young root and cutting vascular systems. Abbreviations: cortex (CO), phloem (P), root (R), root primordium (RP), vascular bundle (VB), vascular cambium (VC), xylem (X). All scale bars = 0.2 mm.

cuttings, were first observed in the leaf-bud cuttings from the root suckers 20 days after planting (Fig. 2.4A), and developed to root primordia penetrating into phloem and cortex (Fig. 2.4B). Roots broke through the epidermis and emerged outside 30 days after planting (Fig. 2.4C), and vascular connections developed between roots and cuttings (Fig. 2.4D).

## Discussion

The phenomenon that the cuttings from the micropropagated trees rooted better than those from grafted trees has been reported on many species, and it was assumed that this improvement in rooting was attributable to rejuvenation caused by micropropagation (Hogue and Neilsen, 1991; Howard et al., 1989; Jones and Webster, 1989; Marks, 1991; Plietzsch and Jesch, 1998; Quamme and Hogue, 1994). The reason why the cuttings from the root suckers of micropropagated trees rooted better than those from the shoots of micropropagated trees is presumably that the former were more rejuvenated than the latter, because root suckers are physiologically more juvenile than shoots (Del Tredici, 1995).

There have been no reports of improvements in rooting of cuttings by shortening of cutting length, except for that of the dwarfing rootstock for Japanese persimmon (Tetsumura et al., 2000a), although the reason why the leaf-bud cuttings survived and rooted better than the other types of the cuttings was unknown. Generally, the use of single-node stem cuttings made rapid propagation from a limited number of stock plants possible (Stoutemyer et al., 1933).

The planting time was important for the rooting of cuttings, as has been reported for some trees and shrubs (Howard, 1996; Murai et al., 1999; Singh et al., 1957; Tetsumura et al., 2000a). The cuttings from the root suckers of both cultivars rooted better when planted in June than in July or August. As suggested by the experiments with ornamental shrubs (Howard, 1996) and the dwarfing rootstock for Japanese persimmon (Tetsumura et al., 2000a), the growth rate of the root suckers probably affected the rooting of the cuttings. The sucker growth decreased in late July when rooting ability declined.

IBA treatment was a prerequisite for the higher rooting of the softwood cuttings from the root suckers, although, in the previous section, IBA treatment did not improve rooting of the hardwood cuttings derived from the etiolated root suckers, which already had root primordia before IBA treatment. IBA treatment was considered to promote the differentiation of root primordia in the Japanese persimmon softwood cuttings as well as in the other fruit tree softwood ones (Gemma et al., 1983; Murai et al., 1999). However,

a further study on IBA treatment method is necessary because the optimum IBA concentration to induce rooting in the cuttings was unclear in this study.

The better survival of cuttings seems to be related to the better rooting (Fig. 2.2). Moreover, the higher humidity in the propagation frame resulted in the better survival of the cuttings from the dwarfing rootstock for Japanese persimmon as well as the better rooting (Tetsumura et al., 2000a). Hence, for the better rooting of the cuttings, the sustenance of their longevity, which was probably influenced by the length and the source of cuttings, the planting time and the environmental condition may be required. Furthermore, active cell division in the cuttings may ensure their better rooting.

This is the first report showing the histological process of adventitious root formation in the softwood cuttings of Japanese persimmon. The origin of root primordia was in the vascular cambium, as was previously reported on European chestnut cuttings (*Castanea sativa* Mill.) (Vieitez et al., 1980). However, the hardwood cuttings from the etiolated root suckers of Japanese persimmon produced root primordia in the phloem in the previous section, and the root primordia of European chestnut shoots treated with etiolation and girdling were also found in the phloem (Biricolti et al., 1994). The origin of root primordia might vary with the treatment for root induction, i. e., IBA treatment or etiolation treatment.

The successful softwood cutting propagation with high rooting percentages and many roots per rooted cuttings suggested that the own-rooted trees of scion cultivars are produced easily, inexpensively, and abundantly by using root suckers and leaf-bud cuttings. Practically, the efficient method for softwood cutting propagation that is reported here would be promising for commercial use, because it did not need cumbersome operations that had been used for softwood cutting propagation of Japanese persimmon (Machida and Fujii, 1969; Murata et al., 1983; Tukamoto et al., 1959). Moreover, it may be applicable to many other persimmon cultivars, because the subsidiary trials with other Japanese persimmons cvs., Fuyu and Hiratanenashi, resulted in successful propagation in the same manner.

## Summary

The factors influencing rooting of softwood cuttings of two cvs., Jiro and Nishimurawase, of Japanese persimmon were studied. The cuttings from the root suckers of micropropagated trees rooted best, followed by those from the shoots of micropropagated trees and grafted trees in that order. Except for the cuttings from grafted trees, the shorter the cuttings, the higher the rooting percentage. When planted in late June, the leaf-bud cuttings taken from the root suckers and treated with IBA

rooted well (70% rooting or more). When planted in late July or late August, however, the leaf-bud cuttings rooted poorly (less than 40% rooting). The leaf-bud cuttings without IBA treatment scarcely rooted when planted in late June, and none of them rooted when planted in late July or late August. There was no significant difference in rooting between the cultivars. Almost all of the leaf-bud cuttings from the root suckers survived during the experimental period (60 days), although the leaf-bud cuttings from the shoots of grafted trees died gradually, and none of the 25-cm cuttings from the root suckers survived 25 days after planting. Vascular cambium of the leaf-bud cuttings from the root suckers began cell division soon after planting. Callus tissue formed in phloem and cortex, and developed extensively. However, active cell division was not observed in the leaf-bud cuttings from the shoots of grafted trees, and the tissues of the 25-cm cuttings from the root suckers did not grow at all. Initial cells of adventitious root in vascular cambium in the leaf-bud cuttings from the root suckers were first observed 20 days after planting, and they developed to roots emerging outside the cuttings 30 days after planting.

## **Chapter 3.**

### **Evaluation of Field Performance of Own-rooted Trees**

#### **Section 1. Evaluation of Micropropagated Trees**

##### **(I) Evaluation of Unpruned Trees**

###### **Introduction**

Japanese persimmon is particularly prone to transplantation shock (Izaki et al., 1958; Kitagawa and Glucina, 1984). Nursery plants grafted on seedling stocks often grow poorly during the first growing season after they are transplanted from outdoor nurseries to orchards. If irrigation is inadequate, poor growth continues for two or more years, and some of them eventually die. This shock results from root pruning and drying during transplanting, and from a lack of lateral and fine roots (Izaki et al., 1958). Tao et al. (1994) reported that micropropagated trees developed many lateral and fine roots, whereas seedlings had one vigorous taproot from which only a few lateral roots elongated, and suggested that micropropagation could alleviate transplantation shock. However, no previous reports compared the growth of grafted trees on seedling stocks with that of micropropagated trees after transplanting. The objective of this sub-section was to evaluate the growth of micropropagated and unpruned Japanese persimmon trees after field establishment in comparison with that of grafted and unpruned trees on seedling stocks.

###### **Materials and Methods**

Three types of 'Nishimurawase' Japanese persimmon trees were studied. Twelve trees grafted on one-year-old seedlings in the spring of 1992 (G) were dug from an outdoor nursery in mid-December 1992, and were heeled-in in moist sand after washing the soil off the roots. Twenty-four micropropagated trees (M), which were derived from shoot tips and maintained in vitro for two years, were obtained as described in Chapter 1-2. One half of them were planted in the nursery in May 1992 and were treated similarly to the grafted trees (MN). The other half were grown in pots until field establishment (MP).

In January 1993, the shoots and roots of three trees of each type were oven-dried at 80 °C for three days and then weighed. The roots were classified into three groups according to their diameters measured after drying.

On 5 March 1993, nine trees of each type were planted in clay loam soil at an

orchard of the Experimental Farm of Kyoto University, and were mulched with black 1.5-m-wide non-woven fabric. A 2.5-cm diameter steel pipe stake was driven beside each tree for training and support, and extended 3.0 m from the soil surface. The within-row spacing was 1.3 m, and the between-row spacing was 6.5 m. The experimental design was a randomized complete block with three replications (trees). In January 1994, 1995, and 1996, one tree of each type was removed from every block, and its shoot and root dry masses were checked as described above. In this manner, the within-row spacing was increased every year. For the trees removed in 1996, the following growth indices were recorded: 1) trunk diameter at 10 cm above the soil surface both at planting and each December; 2) the length and number of annual shoots per tree each November; 3) tree height both at planting time and each November. In 1993-95, each tree received a total of 200, 300, or 750 g of 8N-8P-8K fertilizer per year as a split application. None of the trees were pruned. All flower buds were thinned before flowering. Insecticides and fungicides were applied when necessary.

Except for annual shoot length, all data were subjected to ANOVA and means were separated by Duncan's multiple range test. The data for annual shoot length were subjected to two-way ANOVA. Since the interaction between tree type and year was significant, means were separated by Tukey's honestly significant difference (HSD).

## Results and Discussion

There were considerable differences in size among the types of nursery stock before planting (Fig. 3.1). Hammerschlag and Scorza (1991) pointed out the difficulty of comparing trees grafted on seedling stocks vs. micropropagated trees. All of the nursery stocks used in this study were "one-year-old nursery stocks", whose scions had grown outdoors for one growing season.

Shoots of G trees developed poorly during the first and second growing seasons,

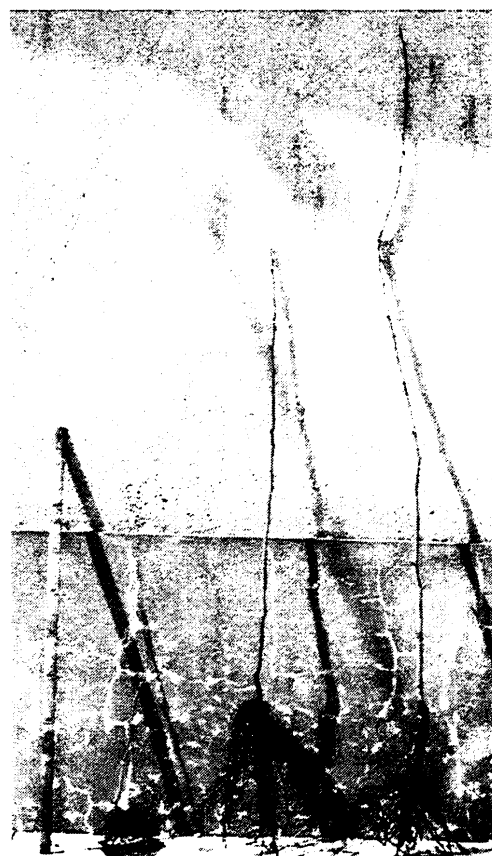


Fig. 3.1. The appearance of 'Nishimurawase' Japanese persimmon nursery stocks before planting (from left to right): micropropagated and raised in pot (MP); micropropagated and raised in outdoor nursery (MN); and grafted on seedling and raised in outdoor nursery (G). Scale bar = 1 m.

as did those of MN trees during the first growing season (Fig. 3.2). However, MP trees fared well soon after field establishment. Ogawa et al. (1994) used mean shoot length per tree as an index of transplantation shock in the nursery stock studies of Japanese persimmon. In this study, although the MP were shorter than the other tree types at planting (Fig. 3.3), the annual shoot length was greater. Therefore, these trees certainly were less affected by transplantation shock. Initially, MP trees had fewer shoots than did the other tree types, because they were smaller and had fewer buds, but no difference was apparent at the end of experiments (data not shown).

The height of G trees increased by only 2% to 4 % of their previous years'

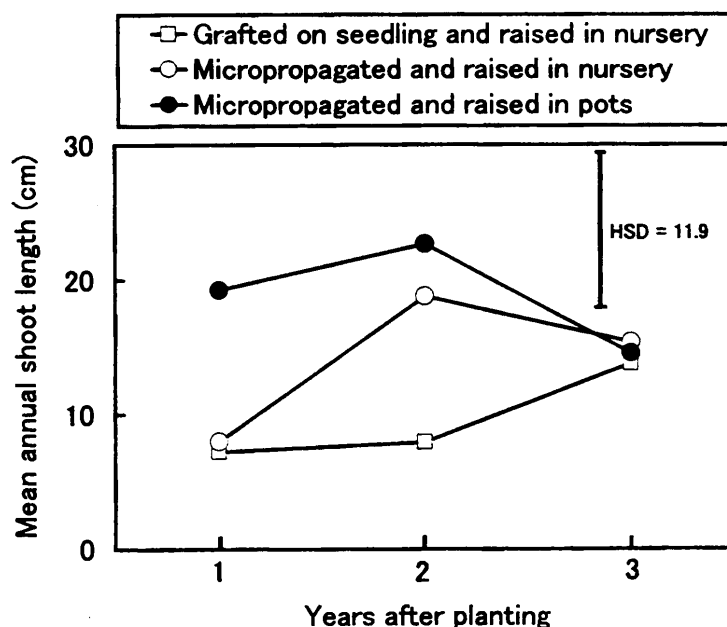


Fig. 3.2. Effect of method of propagation and handling on mean annual shoot length of 'Nishimurawase' Japanese persimmon after planting. Vertical bar = HSD ( $P \leq 0.05$ ). Each point represents a mean,  $n=3$ .

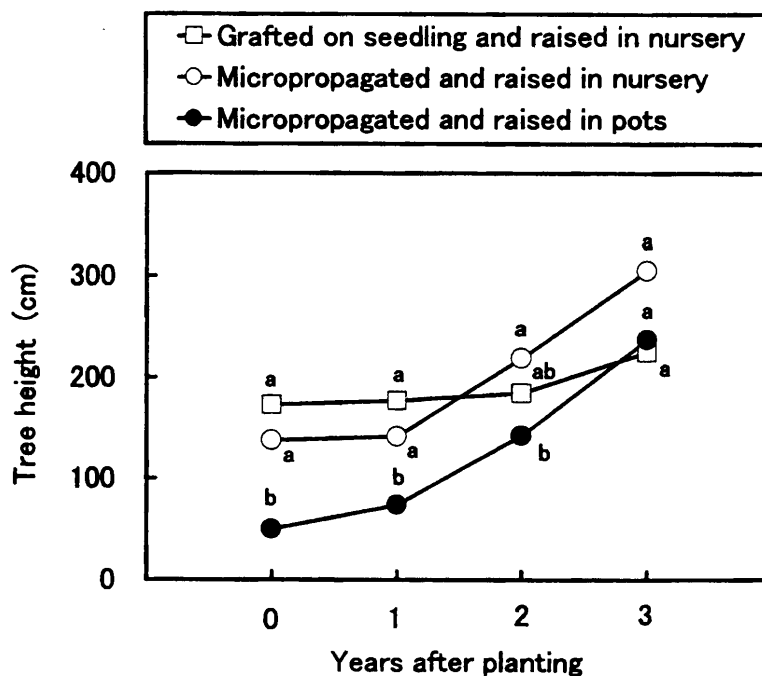


Fig. 3.3. Effect of method of propagation and handling on tree height of 'Nishimurawase' Japanese persimmon after planting. The values for year 0 were measured at planting. Within years, means with the same letter are not significantly different by Duncan's multiple range test at  $P \leq 0.05$ . Each point represents a mean,  $n=3$ .

levels during the first and second growing seasons; the same was true of MN trees during the first growing season. In contrast, the height of MP trees increased by >50% every year (Fig. 3.3). Therefore, although the MP trees were initially one-third as tall as the other tree types, all were the same height by the end of the experiment. In addition, the yearly increase in trunk diameter paralleled the increase in tree height (data not shown).

During the first two growing seasons, the dry mass of the tops of MP trees reached values seven or eight times as great as that of the previous years (Table 3.1). However, the dry masses of the tops of G trees during the first two seasons and MN trees during the first season did not exceed twice the level of the previous years.

Table 3.1. Effects of method of propagating and handling 'Nishimurawase' Japanese persimmon trees on top and root dry mass.<sup>2</sup>

| Method of propagation | Type of nursery | Dry mass of top (g) |        |       |        | Dry mass of roots (g) |       |       |        |
|-----------------------|-----------------|---------------------|--------|-------|--------|-----------------------|-------|-------|--------|
|                       |                 | Year after planting |        |       |        | Year after planting   |       |       |        |
|                       |                 | 0 <sup>3</sup>      | 1      | 2     | 3      | 0                     | 1     | 2     | 3      |
| Grafted on seedling   | Outdoors        | 72.2 a <sup>*</sup> | 93.2 a | 173 a | 801 a  | 116.2 a               | 71 b  | 252 a | 774 a  |
| Micropropagated       | Outdoors        | 41.9 b              | 68.6 b | 525 a | 1201 a | 45.7 b                | 112 a | 746 a | 1072 a |
| Micropropagated       | Pot             | 5.2 c               | 44.2 c | 315 a | 888 a  | 7.6 c                 | 101 a | 416 a | 1050 a |

<sup>2</sup>Data represents mean, n = 3.

<sup>3</sup>Before planting.

<sup>\*</sup>Mean separation within columns by Duncan's multiple range test at  $P \leq 0.05$ .

After the first growing season, the dry mass of the roots of G trees decreased (Table 3.1). Japanese persimmon roots, especially the larger ones, easily become rotten due to root pruning (Maeda and Yoshioka, 1955). In this study, the roots spread out in the nursery. Some roots of the trees were cut off when dug up, and some parts of the large taproots of the seedlings were also cut off. When the seedlings were dug up in January 1994, their taproots had died back to some extent and very few new fine roots had emerged from them. This root damage and underdevelopment may have caused the poor growth of G trees after field establishment. 1-Naphthaleneacetic acid possibly could be applied to overcome transplantation shock by promoting new root development (Izaki et al., 1960).

Before planting, the dry matter of roots of MP trees was distributed more to fine than to middle and large roots (Table 3.2). On the other hand, only 14 % of the root dry



matter of the G trees was in the fine roots. The distribution of the dry matter of roots of MN trees to the fine roots was intermediate between those of the other two tree types. This tendency was consistent with the degree of tree vigor after field establishment. Tree vigor after field establishment could not be estimated from the top-root ratio of nursery stocks, because each of the tree types had similar ratios before planting.

Table 3.2. Effects of method of propagating and handling 'Nishimurawase' Japanese persimmon on distribution of root dry matter before planting in the field.<sup>z</sup>

| Method of propagation | Type of nursery | Dry mass (g)          |          |          | Dry matter distribution (%) |        |       |
|-----------------------|-----------------|-----------------------|----------|----------|-----------------------------|--------|-------|
|                       |                 | Large <sup>y</sup>    | Middle   | Fine     | Large                       | Middle | Fine  |
| Grafted on seedling   | Outdoors        | 69.2±6.7 <sup>x</sup> | 30.2±8.7 | 16.8±2.6 | 61 a <sup>w</sup>           | 25 ab  | 14 b  |
| Micropropagated       | Outdoors        | 12.3±3.0              | 21.7±2.3 | 11.7±2.3 | 26 b                        | 49 a   | 25 ab |
| Micropropagated       | Pot             | 2.3±0.6               | 1.2±0.2  | 4.1±0.2  | 29 b                        | 16 b   | 55 a  |

<sup>z</sup>Data represents mean, n = 3.

<sup>y</sup>Large roots are >5 mm in diameter, middle are 2-5 mm, and fine are <2 mm.

<sup>x</sup>Mean ± SE.

<sup>w</sup>Mean separation within columns by Duncan's multiple range test at  $P \leq 0.05$ .

The results obtained in this study agree with other reports on the transplanting of Japanese persimmon seedling stocks (Izaki et al., 1958; Ogawa et al., 1994), although this is the first study reporting the transplantation of micropropagated trees. During the first few years after field establishment, other micropropagated fruit trees have also exhibited different growth patterns as compared to conventionally propagated (Hammerschlag and Scorza, 1991; Larsen and Higgins, 1990; Zimmerman and Miller, 1991). In order to determine the economic feasibility of micropropagation and to evaluate early field performance, the long-term studies on growth, precocity and yield efficiency of Japanese persimmon are needed, and in the next sub-section, the results are demonstrated in detail.

The results in this sub-section demonstrated that transplantation shock can be alleviated in micropropagated and unpruned Japanese persimmon trees. Currently, Japanese persimmon trees are severely pruned to alleviate transplantation shock. This procedure is unnecessary if MP trees are used. The results in this sub-section also demonstrated that MN trees recovered from transplantation shock sooner than G trees. No significant differences were observed between micropropagated trees and grafted trees on seedling stocks three years after planting.

## **Summary**

The growth of micropropagated and unpruned 'Nishimurawase' Japanese persimmon trees during the initial three years after field establishment was compared with that of grafted and unpruned trees on seedling stocks. Judging from the mean length of annual shoots per tree and the yearly increases in height, trunk diameter, and top and root dry mass, the grafted trees on seedling stocks grew poorly during the first and second growing seasons, while micropropagated trees, raised in an outdoor nursery, developed poorly only during the first growing season. In contrast, micropropagated trees raised in pots fared well soon after field establishment. These trees had more fine than middle and large roots; in contrast, grafted trees on seedling stocks had one large taproot, which died back to some extent after field establishment, with few fine roots.

## **(II) Long-term Evaluation of Pruned Trees**

### **Introduction**

The previous sub-section showed that transplantation shock was reduced in micropropagated trees compared with trees grafted on seedling stocks, although no data on productivity was available.

Zimmerman and Miller (1991) have indicated that the commercial benefits of micropropagated trees can be determined only after several years of field evaluation. Long-term studies have indicated the benefits in apple (Larsen and Higgins, 1993; Quamme and Brownlee, 1993; Rosati and Gaggioli, 1989; Zimmerman and Miller, 1991), blueberry (El-Shiekh et al., 1996), cherry (Quamme and Brownlee, 1993), and peach (Hammerschlag and Scorza, 1991; Quamme and Brownlee, 1993). Micropropagated apple trees produced more fruits than trees on MM106 in the last two years of the five trial years (Rosati and Gaggioli, 1989). The yields of some apple cultivars were greater on micropropagated trees than M 7a (Larsen and Higgins, 1993). Micropropagated apple trees seemed to have been less affected by the nematodes (Zimmerman and Miller, 1991). On the basis of yield and growth, micropropagated peach trees, which showed greater uniformity in crop efficiency than trees on seedlings, should be an acceptable tree type for commercial orchards (Hammerschlag and Scorza, 1991). In colder areas with shorter growing seasons, micropropagated blueberry plants spread more bearing areas than those propagated by cuttings, and produced higher yields (El-Shiekh et al., 1996).

The objective of this sub-section was to evaluate the growth, flowering and fruiting of the micropropagated and pruned Japanese persimmon trees over seven years, and to determine whether they have any commercial advantage over grafted trees.

### **Materials and Methods**

The early-ripening Japanese persimmon cultivar, Nishimurawase, was used in this study. Micropropagated and own-rooted (M) trees obtained by shoot tip culture as described in Chapter 1-2 were planted in 2 L pots in May 1990 and raised until orchard planting. Trees grafted on one-year-old Japanese persimmon seedlings in the spring of 1990 (G) were raised in an outdoor nursery, dug in December 1990, and heeled-in in moist sand until orchard planting. As a result, G trees were initially larger in size than M trees.

In January 1991, the trees were planted in clay loam soil at an orchard of the Experimental Farm of Kyoto University. They received plastic-tube watering as they

needed and were mulched with black 1.5-m-wide nonwoven fabric. A 2.5-cm-diameter steel pipe stake was driven beside each tree for training and support, and extended 3.0 m from the soil surface. The within-row spacing was 1.3 m, and the between-row spacing was 6.5 m. The experimental design was a randomized complete block with four replications comprising four trees each. In March 1993, two trees of each type were removed from every block to prevent overcrowding, and one tree of each type was removed in 1995. In this manner, the within-row spacing was increased to 5.2 m in 1995. Trees were pruned to an open-center shape. Pest and fertilizer management was conducted as is commonly recommended in Japan. All female flowers were removed in years 1 and 2 so as not to deteriorate tree vigor, and fruits were thinned in July thereafter to allow for proper fruit development, as is usually practiced in commercial orchards. Artificial pollination was not conducted.

The trunk cross-sectional area (TCSA) at 10 cm above the soil surface and the tree height were measured in January before pruning, the number of shoots with male and female flowers was counted in May, and the number of shoots, and the mean and total shoot length were measured in November. In 1991, 1992 and 1993, the mean shoot length was measured in the middle of every month during the growing season. Mature fruits with distal skin color of a score of 5 (orange) or more, as determined by the color chart devised for persimmon fruits (Yamazaki and Suzuki, 1980), were harvested and weighed every 5 days. A median harvest date (50% harvest) was calculated as days after the initiation of harvest. The yield efficiency was calculated as the weight (kg) of harvested fruits per  $\text{cm}^2$  of TCSA (Westwood and Roberts, 1970; Fumuro, 1997). On each harvest, the skin color near the calyx, the soluble solids (SS), the number of seeds, and the astringency were measured on five fruits per tree. The means of this fruit quality data were multiplied by the ratios of the fruit number harvested on the date to the total fruit number, and then the total of the transformed values was considered to be the data per tree. SS was measured with a digital refractometer (Atago, Co., Tokyo, Brix 0-32 %), and astringency was evaluated by the tannin print method from the longitudinal section of the fruit (Eaks, 1967).

All data were subjected to ANOVA. Percentage data were subjected to arcsin transformation before ANOVA.

## Results

M trees, which were not affected by transplantation shock, fared well after orchard establishment. On the other hand, G trees in 1992 were less tall than in 1991 (Fig. 3.4A), because the leaders of G trees were cut back severely at planting to

alleviate transplantation shock but they did not grow well during the first growing season. No TCSA increment was observed for G trees during the first growing season, probably because of transplantation shock. In 1992, there was no significant difference between the tree types in TCSA, although that of G trees was larger than M trees in 1991 (Fig. 3.4B). After 1992, both of the tree types grew constantly. However, M trees grew more vigorously than G trees; hence, M trees were significantly higher after the 5th growing season and larger in TCSA after the 4th growing season than G trees. The difference in TCSA increased with the passage of the growing season.

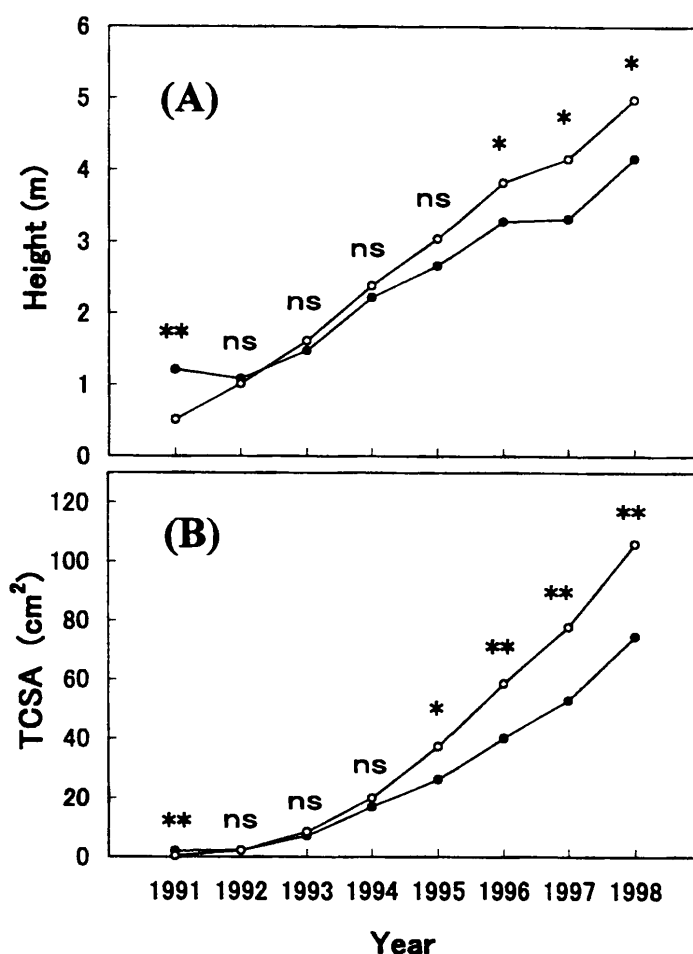


Fig. 3.4. Comparison of tree height (A) and TCSA (B) of M (○) and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1991. ns, \*, \*\* Nonsignificant or significant at  $P < 0.05$  or 0.01, respectively, by ANOVA.

The total shoot length and the number of shoots of M trees were significantly greater than those of G trees after the 4th year (Fig. 3.5A and B). The mean shoot length of M trees was greater than that of G trees every year, and there were significant differences observed in most of the years (Fig. 3.5C). In 1991, the difference was more than 10 cm because of the effect of transplantation shock on G trees. Fig. 3.6 shows the typical growth pattern of the shoots during the growing season, and demonstrates that the difference between the tree types was apparent after mid-June. Most of the shoots stopped growing before the beginning of June, but some of them grew continuously during the growing season, and some made secondary growth from June. Therefore, the difference in the shoot length comes from the difference in the proportion of these vigorous shoots to the total ones.

In the first and the second years, 13% and 38%, respectively, of G trees bore female flowers compared to 19% and 6% of M trees, although in both years there was no significant difference in the percentage of trees with female flowers between the tree types ( $P < 0.05$ ). After the third year, all of the trees bore female flowers. G trees had more shoots with female flowers than M trees in the second and the third years, although there was no significant difference in the first year (Fig. 3.7A). The difference in the number of shoots with female flowers disappeared in the 4th and 5th years, and M trees had more in the 6th year. In the 7th year, both of the tree types had more than 150 shoots with female flowers.

In the first year, 19% of G trees bore male flowers compared to 31% of M trees, but there was no significant difference between the tree types ( $P < 0.05$ ). In the second year, 75% of both of the tree types bore male flowers, and 100% bore them after the 5th year. Except for the first year,

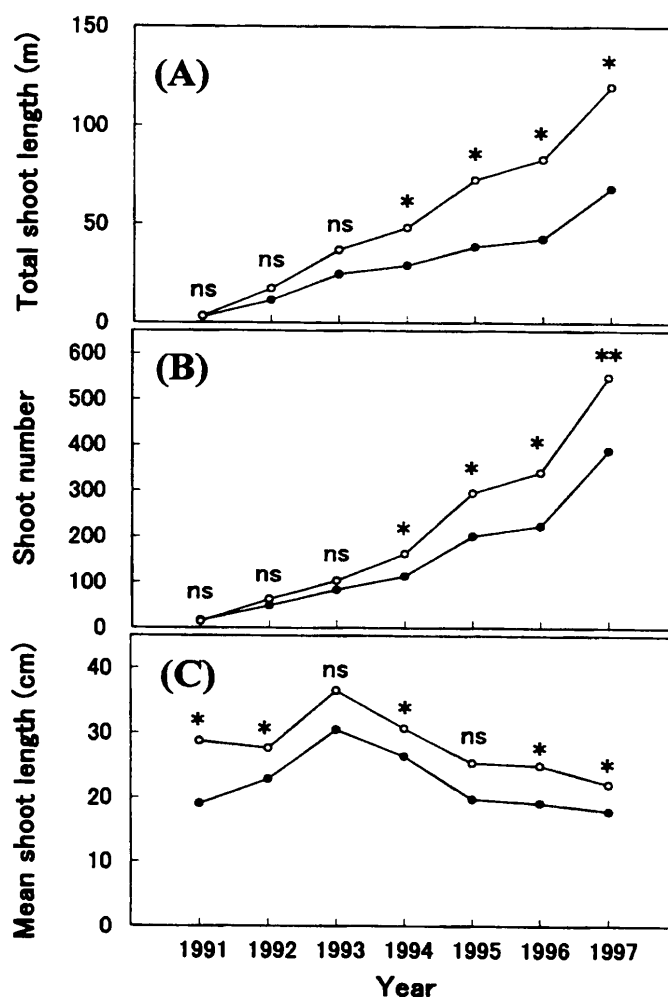


Fig. 3.5. Comparison of total shoot length (A) number of shoots (B), and mean shoot length (C) of M (○) and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1991. ns, \*, \*\* Nonsignificant or significant at  $P < 0.05$  or  $0.01$ , respectively, by ANOVA.

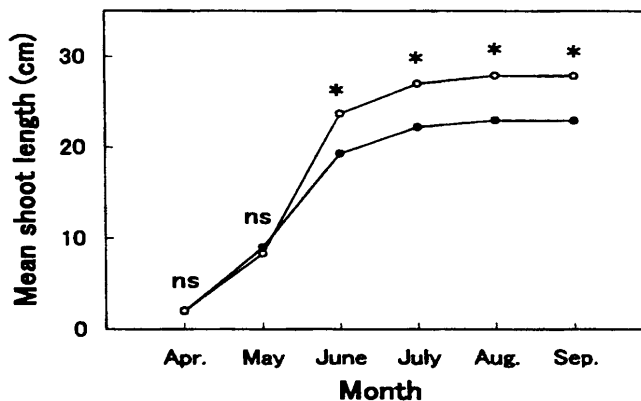


Fig. 3.6. Monthly increase in mean shoot length of M (○) and G (●) trees of 'Nishimurawase' Japanese persimmon in the second growing season (1992). ns, \* Nonsignificant or significant at  $P < 0.05$  by ANOVA.

G trees had more shoots with male flowers than M trees (Fig. 3.7B). However, there were no significant differences noted, because some of G trees bore male flowers abundantly while others bore them as slightly as M trees.

The yield changed with the number of shoots with female flowers. In 1993, the first harvest year, G trees produced more fruit than M trees (Fig. 3.8A). However, they produced nearly the same amount in 1994 and 1995. In addition, M trees produced more than G trees in 1996, the off-year. In 1997, M trees produced more fruit but the difference was not found to be significant. The

cumulative yield for five years of M trees was higher than that of G trees due to the yields in 1996 and 1997, but the difference was not significant. Except for the off-year, G trees produced more efficiently than M trees every year and totally, but significant differences were only observed in 1993 and 1994 (Fig. 3.8B).

M trees did not bear any variant fruit, and no consistent difference in fruit quality between the tree types could be observed. The average fruit weight of both of the tree types was between 140- and 160-g, except for 113 - 114 g in 1994, in which summer it was unusually hot and dry. Furthermore, no significant difference was observed between the tree types every year, except for 1995, in which the fruit of M trees was heavier than that of G trees by 12 g. The seed number per fruit, SS and the percentage of non-astringent fruits varied (2.9 - 5.5 seeds, 13.5 - 17.7% and 57 - 94%,

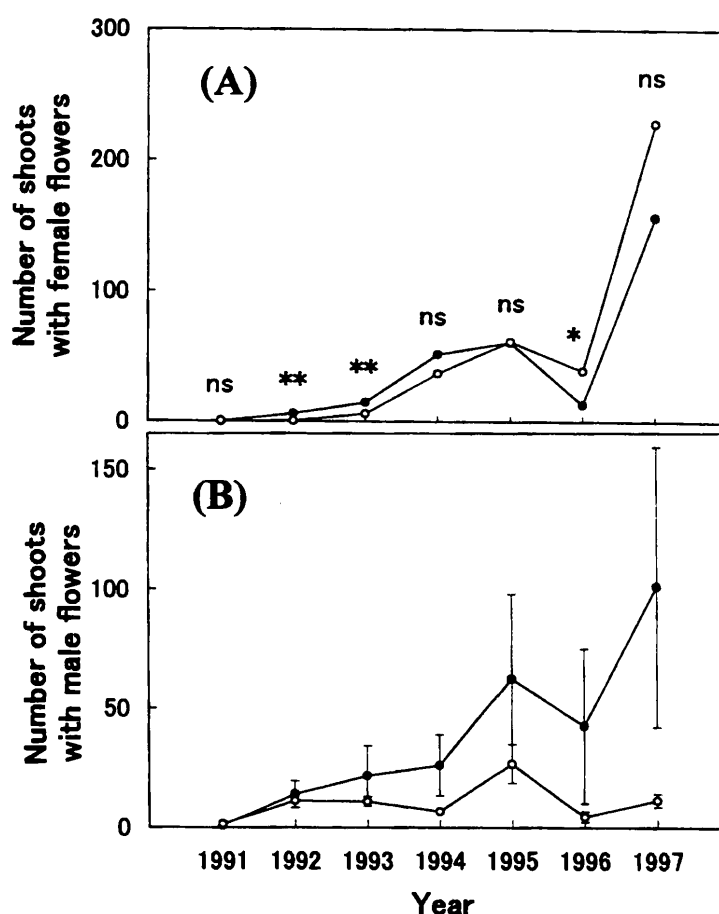


Fig. 3.7. Comparison of number of shoots with female flowers (A) and with male flowers (B) of M (○) and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1991. ns, \*, \*\* Nonsignificant or significant at  $P < 0.05$  or  $0.01$ , respectively, by ANOVA (A). There was no significant difference between M and G trees for each year ( $P < 0.05$ ) and vertical bars represent standard errors (B).

respectively) with the years, but no significant difference between the tree types was observed. The skin color near the calyx, one of the indices of fruit ripening of early-ripening Japanese persimmon cultivars, showed that the fruit of M trees ripened earlier than that of G trees in the first and second harvest years. After the third harvest year, however, no difference was observed. There was no significant difference in the median harvest date between the tree types every year, except for the first harvest year, when that of M trees was 4 days earlier than G trees.

## Discussion

Tree size at planting is important for tree growth during the initial few years of orchard establishment, but Hammerschlag and Scorza (1991) have pointed out the difficulty of comparing trees grafted on seedling stocks vs. micropropagated trees. Although there was a considerable difference in size between the types of nursery stock before planting, all of the nursery stocks used in this study were “one-year-old nursery stocks”, whose scions had grown outdoors for one growing season, as described in the previous subsection.

As shown in the previous sub-section, transplantation shock severely affected the trees grafted on Japanese persimmon seedlings for two years. This weakness of the grafted trees was also confirmed in this

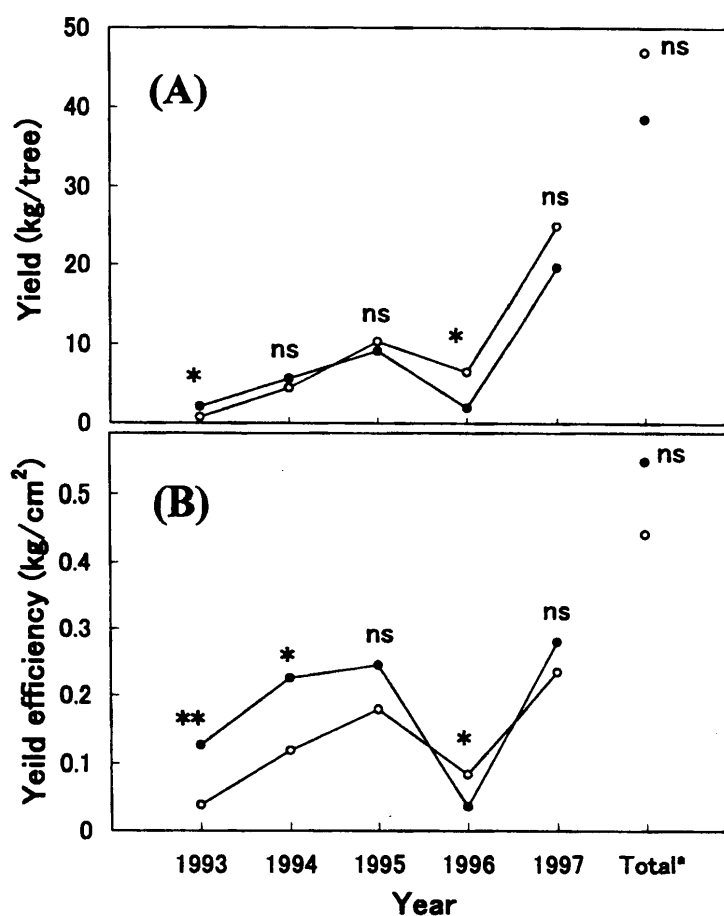


Fig. 3.8. Comparison of yield (A) and yield efficiency (B) of M (○) and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1991. ns, \*, \*\* Nonsignificant or significant at  $P < 0.05$  or  $0.01$ , respectively, by ANOVA. \* Cumulative yield 1993-1997 (A), and cumulative yield 1993-1997 / final TCSA (B).



study, but the transplantation shock occurred for only one year. Pruning that was not carried out in the previous sub-section probably alleviated transplantation shock to some extent because cultural procedures in Japan recommend that Japanese persimmon trees be severely pruned for the purpose of alleviating transplantation shock.

The micropropagated apple trees have proved to often grow vigorously (Jones and Hadlow, 1989; Rosati and Gaggioli, 1989; Zimmerman and Miller, 1991), and sometimes even develop better than trees grafted on seedlings (Larsen and Higgins, 1993), while the micropropagated plants of blueberry (El-Shiekh et al., 1996; Grout et al., 1986) and blackberry (Swartz et al., 1983) have also been known to grow very well. As for 'Nishimurawase' Japanese persimmon, M trees also grew more vigorously than G trees even seven years after orchard establishment.

In Japan, the shoot elongation of Japanese persimmon trees has been reported to stop between late-May and early-June (Nii, 1980; Harada, 1984), and flower initiation has begun in early July (Harada, 1984). Most shoots of 'Nishimurawase' trees in this study stopped growing in early June, but some vigorous shoots, especially those of M trees, elongated continuously or made a secondary growth as soon as they stopped growing. Shoots that have made a second or a third growth have hardly ever born female-flower buds (Sobajima, 1979). Therefore, the fact that the number of shoots with female flowers of M trees was less than that of G trees in the initial few years, except for the first year, when G trees were severely pruned and affected by the transplantation shock, was possibly caused by the fact that some of M trees had more vigorous shoots than G trees. As has been suggested by Tao et al. (1994), reinvigoration or partial rejuvenation (Pierik 1990), but not true rejuvenation, may have occurred in M trees, because some of them bore flowers during the first growing season.

'Nishimurawase' is not normally used as a pollinizer in Japan, because it does not bear many male flowers and the amount of pollen produced is not enough. Therefore, it is desirable that it should bear less male flowers so as not to deteriorate tree vigor. From this point of view, M trees are preferable because they will hardly produce male flowers while some G trees will produce plenty of them for a long time owing to the sex determination habit of monoecious-type Japanese persimmon cultivars (Yonemori et al., 1993). Fumuro (1992) showed that the micropropagated young 'Nishimurawase' trees bore less male flowers than the adult trees grafted on seedling rootstocks.

The yield probably increased more rapidly in M trees than in G trees, because M trees grew faster than G trees and not because the yield efficiency was higher in M trees. In colder areas with shorter growing seasons, the micropropagated blueberry plants

have spread more bearing areas than those propagated by cuttings, and have produced higher yields (El-Shiekh et al., 1996). M trees might be more useful for commercial orchard production when planted in colder areas and/or less fertile soil, because the secondary shoot growth of persimmon trees may be owing to high soil fertility or mild climatic conditions (Kitagawa and Glucina, 1984). It was demonstrated that the differences in the productivity and the tree vigor of micropropagated apple and peach trees existed between the different growing sites (Hammerschlag and Scorza, 1991; Zimmerman and Miller, 1991).

The leaves and the bark of M trees were true-to-type, as well as fruit shape and quality. Seed number is an important factor in a pollination variant, non-astringent cultivar such as 'Nishimurawase', because it is only the well-seeded fruit that loses its astringency and has marketability ; and there was no problem related to the fruit of M trees in regard to astringency and quality. In addition, 'Nishimurawase' is an early-ripening cultivar, so the harvesting time is an important factor. In the first few harvest years, the fruit of M trees ripened earlier than that of G trees. However, there was no difference observed in the ripening time between the tree types after the third harvest year.

Larsen and Higgins (1993) have concluded that the orchard performance of micropropagated apple trees is a highly cultivar dependent. It is necessary to evaluate the long-term orchard performance of micropropagated and own-rooted trees of each Japanese persimmon cultivar before their commercial use can be determined. In preliminary experiments, micropropagated trees of 'Hiratanenashi' and 'Jiro' Japanese persimmons also showed more vigorous growth in the orchard than those grafted on seedlings. As with micropropagated apple trees (Zimmerman and Steffens, 1995), therefore, the micropropagated and own-rooted Japanese persimmon are unlikely to successfully substitute for trees on dwarfing or semi-dwarfing rootstock. However, at some orchards with less fertile soil and/or in colder area, M trees should have advantages over the conventionally propagated trees, because they establish more easily and grew faster.

## **Summary**

Growth habit, precocity and intensity of flowering and fruiting, and fruit quality of micropropagated (M) and pruned 'Nishimurawase' Japanese persimmon trees over seven years after orchard establishment were compared with those of trees grafted on seedling stocks (G). Judging from the yearly increase in tree height and trunk cross-sectional area, M trees fared well without transplantation shock and grew more

vigorously than G trees. The vigorous growth of M trees made the total and mean shoot length greater, and the number of shoots larger. The difference in the mean shoot length between the tree types appeared after mid-June because some vigorous shoots, especially of M trees, elongated continuously or made a secondary growth, although most of the shoots stopped growing before the beginning of June. In the first few years but the first one, G trees had more shoots with female flowers than M trees, although the difference disappeared in the 4th and the 5th years. Inversely, M trees had more in the 6th year (off-year), but the difference disappeared again in the 7th year. Except for the first year, G trees had more shoots with male flowers than M trees, but there was no significant difference. The yield changed with the number of shoots with female flowers. Except for the off-year, G trees produced fruit more efficiently than M trees, but significant difference was observed only in the first and the second harvest years. M trees did not bear any variant fruit, and no consistent difference in the fruit quality (average weight, number of seed, soluble solids and astringency) was observed between the tree types. These results suggested that M trees would be advantageous for some commercial orchards where initial vigorous vegetative growth is preferable, such as orchards with less fertile soil and/or in colder areas.

## **Section 2. Evaluation of Trees Propagated by Cuttings**

### **Introduction**

The previous section showed that transplantation shock was reduced in the micropropagated and own-rooted Japanese persimmon trees compared with the trees grafted on seedling, but the former grew more vigorously than the latter even seven years after establishment, so that the former were unlikely to successfully substitute for trees on dwarfing or semi-dwarfing rootstock. As for apple, micropropagated dwarfing rootstocks with scions 'Greensleeves' showed vigorous shoot growth and delayed cropping in comparison with their conventionally propagated counterparts (Jones and Hadlow, 1989). However, Jones and Webster (1993) overcame the problems with the micropropagated dwarfing rootstocks by using rootstocks from improved conventional propagation from micropropagated plants, which were apparently rejuvenated and had much improved capacity for vegetative propagation.

Chapter 2 demonstrated the efficient vegetative production of own-rooted Japanese persimmon trees by cutting propagation. On the other hand, there have apparently been no reports of field performance of own-rooted trees of Japanese persimmon cultivar propagated by cuttings, although there were a few reports of propagation of Japanese persimmon cultivars by cuttings (Machida and Fujii, 1969; Murata et al., 1983). The objective of this section was to evaluate the early field performance of own-rooted Japanese persimmon trees propagated by hardwood cuttings in comparison with those of grafted trees on seedling stocks and micropropagated trees.

### **Materials and Methods**

Three types of 'Nishimurawase' Japanese persimmon trees were studied. Three trees grafted on one-year-old seedlings in the spring of 1996 (G) were dug from an outdoor nursery in mid-December 1996, and were heeled-in in moist sand after washing the soil off the roots. Three micropropagated and own-rooted trees (M), which were derived from shoot tips and maintained in vitro for six years, were obtained as described in Chapter 1-2, planted in the nursery in July of 1996, and treated in the similar way to the grafted trees. Three own-rooted trees propagated by hardwood cuttings (C) were obtained as described in Chapter 2-1, and grown in the propagation baskets until field establishment.

In January 1997, the shoots and roots of three trees of each type were oven-dried at 80 °C for three days and then weighed. The roots were classified into three groups according to their diameters measured after drying.

On 5 March 1997, three trees of each type were planted in clay loam at an orchard of the Experimental Farm of Kyoto University, and were mulched with black 1.5-m-wide non-woven fabric. A 2.5-cm diameter steel pipe stake was driven beside each tree for training and support, and extended 3.0 m from the soil surface. The within-row spacing was 5.5 m, and the between-row spacing was 6.5 m. The experimental design was a randomized complete block with one replication (tree).

The trees were pruned to a modified central leader type. Pest and fertility management was conducted as is commonly recommended in Japan. All female flowers were removed in 1997 and 1998, and fruits were thinned in July 1999. Artificial pollination was not conducted.

Tree height, tree coverage, tree volume, and trunk cross-sectional area (TCSA) were measured in January before pruning. The number of shoots with male and female flowers was counted in May. The number of shoots and the mean and total shoot length were measured in November. Mature fruit with distal skin color of a score of 5 (orange) or more, as determined by the color chart devised for persimmon fruits (Yamazaki and Suzuki, 1980), were harvested and weighed every 6 days. The yield efficiencies were calculated as the weight (kg) of harvested fruits per  $\text{cm}^2$  of TCSA, per  $\text{m}^2$  of tree coverage, and per  $\text{m}^3$  of tree volume. On each harvest, the skin color near the calyx, the soluble solids (SS), the number of seeds, and astringency were measured on five fruits per tree. The means of this fruit quality data were multiplied by the ratios of the fruit number harvested on the date to the total fruit number, and then the total of the transformed values was considered to be the data per tree. SS was measured with a digital refractometer (Atago, Co., Tokyo, Brix 0-32 %), and astringency was evaluated by the tannin print method from the longitudinal section of the fruit (Eaks, 1967).

All data were subjected to ANOVA, and means were separated by Fisher's protected least significant difference (FLSD). Percentage data were subjected to arcsin transformation before ANOVA.

## Results and Discussion

Before planting, C trees did not have any large roots, and most of the dry matter of their roots was distributed to fine roots (Table 3.3). On the other hand, only 6% of the root dry matter of G trees was distributed to fine roots. The distribution of the dry matter of roots of M trees to the fine roots was intermediate between those of the other two tree types. Each of the tree types had a similar top-root ratio, although G trees were the largest in shoot and root dry matter (data not shown).

C trees, which were not affected by transplantation shock, fared well after

Table 3.3. Effects of method of propagating 'Nishimurawase' Japanese persimmon trees on distribution of root dry matter before planting in the field.<sup>2</sup>

| Method of propagation | Dry mass (g)             |            |            | Dry matter distribution (%) |        |      |
|-----------------------|--------------------------|------------|------------|-----------------------------|--------|------|
|                       | Large <sup>3</sup>       | Middle     | Fine       | Large                       | Middle | Fine |
| Grafting on seedling  | 90.5 ± 13.9 <sup>*</sup> | 28.8 ± 3.3 | 6.8 ± 0.4  | 71 a <sup>w</sup>           | 24 b   | 6 c  |
| Micropropagation      | 5.9 ± 1.6                | 7.4 ± 0.9  | 3.1 ± 0.5  | 35 b                        | 45 a   | 20 b |
| Cutting               | 0.0                      | 3.0 ± 0.9  | 20.3 ± 4.4 | 0 c                         | 12 b   | 88 a |

<sup>2</sup>Data represents mean, n = 3.

<sup>3</sup>Large roots are >5 mm in diameter, middle are 2-5 mm, and fine are <2 mm.

<sup>\*</sup>Mean ± SE.

<sup>w</sup>Mean separation within columns by FLSD at *P* < 0.05.

planting (Fig. 3.9A). M trees were not affected by transplantation shock either, although they had less fine roots than C trees, partly because the leaders of M trees were cut back severely at planting to alleviate transplantation shock. On the other hand, G trees in 1997 were less tall than in 1998, because the leaders were also cut back but they did not grow well during the first growing season, in the same way as in Chapter 3-1-II. Although C trees were the shortest in tree height at planting, they grew well and reached the same height as M trees two years after planting. G trees also grew as high as M trees three years after planting, and this result

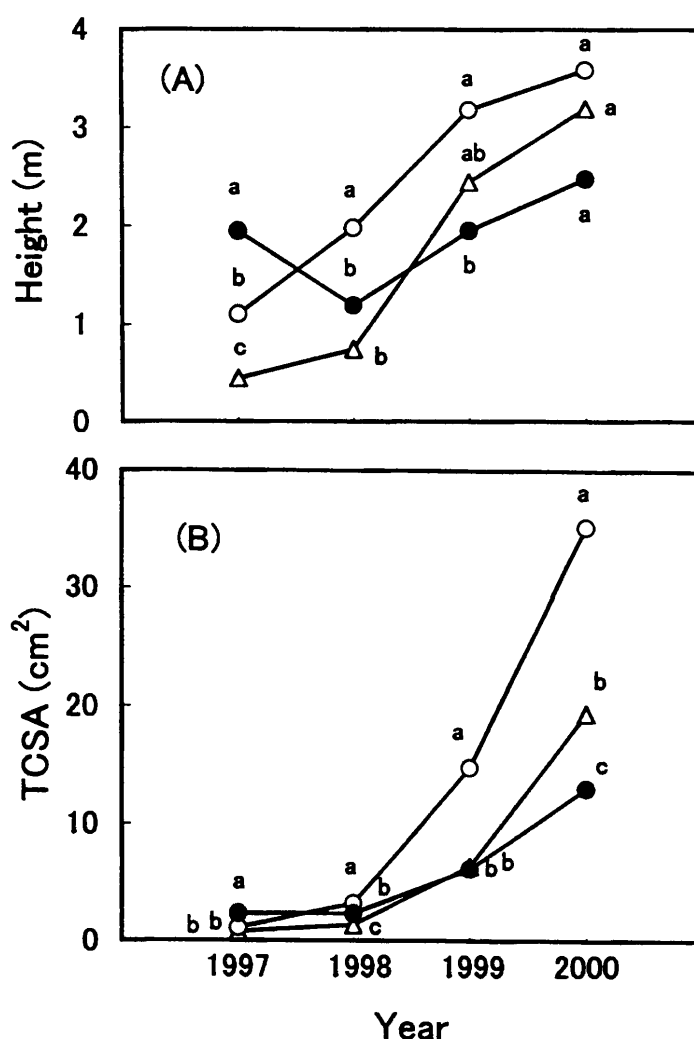


Fig. 3.9. Comparison of tree height (A) and TCSA (B) of C (Δ), M (○), and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1997. Within years, means with the same letter are not significantly different by FLSD at *P* < 0.05. Each point represents a mean, n = 3.

was different from that in Chapter 3-1-II, possibly because of the difference in training and number of replication.

TCSA increment in M trees was the most rapid, while that in G trees was the slowest (Fig. 3.9B). TCSA of C trees was the smallest at planting and one year after planting, but it became larger than that of G trees three years after planting. TCSA of Japanese persimmon tree was highly correlated with dry weight of scion and rootstock (Fumuro, 1999). Therefore, TCSA increment after planting suggested that vigor of C trees was intermediate between those of G and M trees.

The total shoot length and the number of shoots of M trees were significantly greater than those of the other two tree types during the experiments, and their yearly increase was rapid (Fig. 3.10A and B). These results showed probably that the vigor of C trees was less

than that of M trees, although the mean shoot length of M trees was the same as that of the C trees during the experiments (data not shown).

C trees did not bear female flowers in the first and the second years. In the third year, C trees had the lowest number of shoots with female flowers, but the percentage of shoots with female flowers in total shoots was the same as that of M trees (Table 3.4). One of C trees bore male flowers in the second year, and all C trees bore male flowers in the third year. C trees bore male flowers as well as G trees in the third year, although M trees did not bear at all (Table 3.4). However, the differences in the number and the percentage of shoots with male flowers were not significant, because the differences

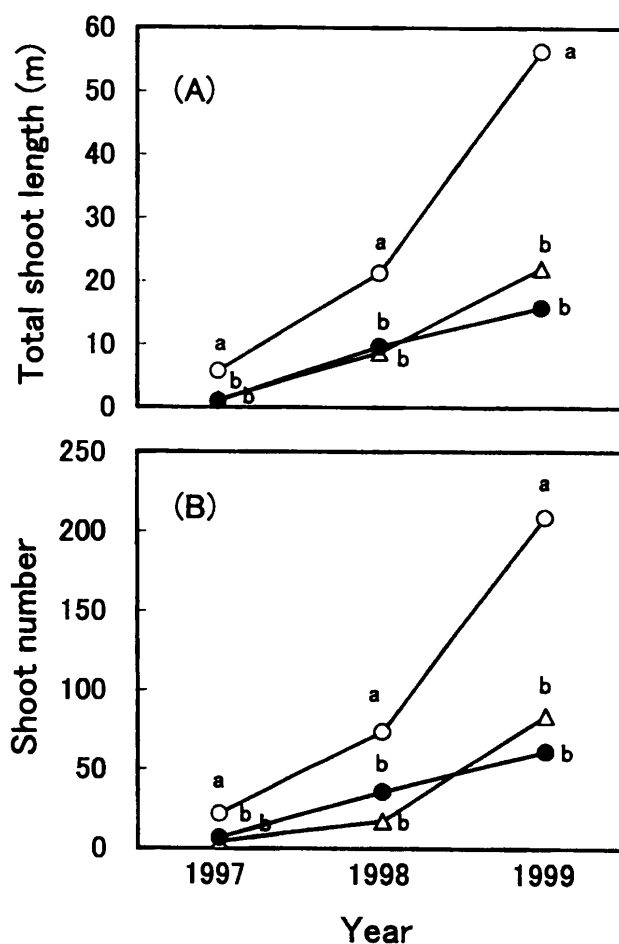


Fig. 3.10. Comparison of total shoot length (A) and number of shoots (B) of C (Δ), M (○), and G (●) trees of 'Nishimurawase' Japanese persimmon planted in January 1997. Within years, means with the same letter are not significantly different by FLSD at  $P < 0.05$ . Each point represents a mean,  $n = 3$ .

among G trees were large. These results of flowering suggested that C trees differed from both G and M trees.

Table 3.4. Effects of method of propagating 'Nishimurawase' Japanese persimmon trees on number and percentage of shoots with flowers three years after planting.<sup>z</sup>

| Method of propagation | No. of shoots with female flowers | % of shoots with female flowers <sup>y</sup> | No. of shoots with male flowers | % of shoots with male flowers <sup>x</sup> |
|-----------------------|-----------------------------------|--|---------------------------------|--|
| Grafting on seedling  | 18.3 a <sup>w</sup>               | 31 a   | 7.0 a                           | 11 a                                       |
| Micropropagation      | 15.3 a                            | 7 b  | 0 a                             | 0 a  |
| Cutting               | 8.7 b                             | 8 b  | 12.0 a                          | 13 a                                       |

<sup>z</sup>Data represents mean, n = 3.

<sup>y</sup>Percentage of shoots with female flowers in total shoots.

<sup>x</sup>Percentage of shoots with male flowers in total shoots.

<sup>w</sup>Mean separation within columns by FLSD at  $P < 0.05$ .

In the first harvest year, the relationships of the yield and the yield efficiencies between tree types were similar to those of the number and the percentages, respectively, of shoots with female flowers (Table 3.5). C trees produced less fruit than the other two tree types, but the yield efficiencies of C trees were the same as those of M trees. C trees did not bear any variant fruit, and no consistent difference in fruit quality among the tree types was observed (data not shown). The leaves and the bark of C trees were true-to-type, as well as those of M trees.

Table 3.5. Effects of method of propagating 'Nishimurawase' Japanese persimmon trees on yield and yield efficiencies in the first harvest year (three years after planting).<sup>z</sup>

| Method of propagation | Yield (kg/tree)    | Yield efficiency                 |  |  |
|-----------------------|--------------------|----------------------------------|--|--|
|                       |                    | Yield/TCSA (kg/cm <sup>2</sup> ) | Yield/Tree coverage (kg/m <sup>2</sup> ) | Yield/Tree volume (kg/m <sup>3</sup> ) |
| Grafting on seedling  | 2.8 a <sup>y</sup> | 0.21 a                           | 2.6 a                                    | 1.9 a                                  |
| Micropropagation      | 2.3 a              | 0.06 b                           | 0.6 b                                    | 0.2 b                                  |
| Cutting               | 1.3 b              | 0.06 b                           | 0.6 b                                    | 0.3 b                                  |

<sup>z</sup>Data represents mean, n = 3.

<sup>y</sup>Mean separation within columns by FLSD at  $P < 0.05$ .

Judging from the increase in TCSA, total shoot length, and shoot number, C trees grew less vigorously than M trees. However, because C trees were the smallest at planting, it is reasonable to assume that they were planted too small to be accurately compared with the other two types and grew vigorously as M trees after planting. In this study, although there was a considerable difference in size among the nursery stock



types, all of the nursery stocks used were "one-year-old nursery stocks", whose scions had grown outdoors for one growing season, in the same way as described in the previous section. Male flowering suggested, however, that C trees were different from M trees.

Field performance of fruit trees propagated by cuttings was different from that propagated by grafting on seedling rootstocks, and this difference varied with the species and the cultivars. Peach trees (*Prunus persica* (L.) Batsch) of several cultivars propagated by semi-hardwood cuttings had a growth rate comparable to that of budded trees (Couvillon and Erez, 1980), but they had better leaf elemental content than those on various seedling rootstocks (Couvillon, 1982). Own-rooted 'Stanley' plum trees (*P. domestica* L.) were smaller than 'Stanley' on Myrobalan plum seedlings (*P. cerasifera* Ehrh.) (Okie, 1987). One-year-old nursery stocks of 'Ina Bungo' mume (*P. mume* Sieb. et Zucc.) propagated by hardwood cuttings were comparable in size to two-year-old nursery stocks grafted on seedlings (Kumashiro and Kobayashi, 1986). However, mume trees propagated by cuttings are widely used for bonsai because they become compact (Murai et al., 1999). In this study, judging from the increment in TCSA, C trees grew more vigorously than G trees, so that the former were unlikely to successfully substitute for trees on dwarfing rootstock. However, further observations of their field performance in comparison with those of the other tree types will be needed to examine the growth rate of adult trees and the productivity in high productive age.

## Summary

Growth habit, precocity and intensity of flowering and fruiting, and fruit quality of 'Nishimurawase' Japanese persimmon trees propagated by hardwood cuttings (C) over three years after orchard establishment were compared with those of trees grafted on seedling stocks (G) and micropropagated (M). Most of the roots that C trees had at planting were fine ones. The change in tree height after planting showed that C and M trees fared well without transplantation shock but G trees did not. Judging from the yearly increase in TCSA, total shoot length, and number of shoots, C trees grew less vigorously than M trees. Judging from the yearly increase in TCSA, C trees grew more vigorously than G trees. C trees did not bear female flowers in the first and the second years. In the third year, the first harvest year, C trees had the lowest number of shoots with female flowers and produced the lowest amount of fruit, but the percentage of shoots with female flowers in total shoots and the yield efficiencies were the same as those of M trees. C trees had shoots with male flowers as well as G trees in the third year, while none of M trees bore male flowers. C trees did not bear any variant fruit, and

no consistent difference in the fruit quality (average weight, number of seed, soluble solids and astringency) was observed among the tree types. These results suggested that field performance of C trees was not the same as those of M or G trees, although further long-term evaluation is needed to reveal the character of the adult trees.

## Conclusions and Prospects

This study was designed to produce the own-rooted trees of Japanese persimmon cultivars by micropropagation and cutting propagation, and then to evaluate their field performance in comparison with the conventionally propagated (grafted on seedlings) trees.

In Chapter 1, the *in vitro* rooting ability of Japanese persimmon difficult-to-root cultivars was improved by long-term subculturing in the BA supplemented medium. More improvements were made by using the adventitious shoots forming on *in vitro* roots. The longer roots easily formed the adventitious shoots on MS medium supplemented with 10  $\mu$  M zeatin and 0.01  $\mu$  M IAA, and these shoots from easy-to-root cultivars as well as difficult-to-root cultivars rooted and were acclimatized better than those derived from shoot tips. No obvious variants of these regenerants were observed, probably because they were differentiated from the pericycle of roots. As for acclimatization, micropropagules survived after transplanting to the pots only when cultured in the rooting medium after the rooting treatment, and the optimum temperature and light conditions improved the survival and the growth of micropropagules.

In Chapter 2, the hardwood cuttings collected from basal part of root suckers forming on roots of micropropagated trees rooted well, especially when mounded at their base until early summer in a similar way as stool-layering. Before the rooting treatment, adventitious root primordia were found in the basal part of the cuttings from basal part of mounded root suckers. The softwood cuttings collected from root suckers of micropropagated trees rooted better than those from shoots of micropropagated trees and grafted trees, and the shorter cuttings rooted better than the longer cuttings. The rooting treatment with IBA and the earlier planting time were prerequisites for their higher rooting. The softwood cuttings with higher rooting ability showed the active cell division soon after planting.

In Chapter 3, the unpruned trees that were micropropagated and were raised in pots were not affected by transplantation shock, and fared well soon after planting. In contrast, the micropropagated trees raised in an outdoor nursery were affected by transplantation shock for one growing season, and the trees grafted on seedlings were affected by transplantation shock for two growing seasons. The micropropagated and pruned trees grew more vigorously than the pruned trees on grafted seedlings seven years after planting. The grafted trees produced more fruits than the micropropagated trees in the initial harvest years, but the latter produced more after the third harvest year.

Except for the off-year, the grafted trees produced fruit more efficiently than the micropropagated trees. The micropropagated trees did not bear any variant fruit, and no consistent difference in the fruit quality was observed between the tree types. The trees propagated by hardwood cuttings fared well after planting without transplantation shock, and grew less vigorously than the micropropagated trees and more vigorously than the grafted trees. In the first harvest year, they produced the lowest amount of fruit, although their yield efficiency was the same as that of the micropropagated trees.

This study showed how to produce the own-rooted trees of Japanese persimmon cultivars by micropropagation and cutting propagation. However, their field performance might not be suitable for the demands of Japanese persimmon growers who encounter the difficulties in growing large trees, because they are unlikely to successfully substitute for trees on dwarfing rootstock. In order to make the dwarfed trees of Japanese persimmon cultivars, cutting propagation of dwarfing rootstocks is an appropriate method, since the trees grafted on micropropagated dwarfing rootstocks often showed negative characteristics (Jones and Hadlow, 1989; Jones and Webster, 1993). Cutting propagation of some dwarfing rootstocks for Japanese persimmons has been successful by using the cuttings collected from their root suckers (Tetsumura, 2000; Tetsumura et al., 2000a; Tetsumura et al., 2000b), and the field performance of the trees grafted on dwarfing rootstocks are now evaluated.

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## **Publication List**

### **Chapter 1-1 (I)**

Tetsumura, T. 1997. Effect of types of cytokinin used for in vitro shoot proliferation of Japanese persimmon on the subsequent rooting of shoots. *Acta Hort.* 436:143-147.

### **Chapter 1-1 (II) (a)**

Tetsumura, T. and H. Yukinaga. 1996. High-frequency shoot regeneration from roots of Japanese persimmon. *HortScience* 31(3):463-464.

### **Chapter 1-1 (II) (b)**

Tetsumura, T. and H. Yukinaga. 2000. Comparative rooting of shoot tips of four Japanese persimmon cultivars vs. shoots regenerated from roots cultured in vitro. *HortScience*. 35(5):940-944.

### **Chapter 1-2**

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### **Chapter 2-1**

Tetsumura T., R. Tao, and A. Sugiura. 2001. Factors affecting rooting of Japanese persimmon hardwood cuttings. *J. Jpn. Soc. Hort. Sci.* 70(2):(in press).

### **Chapter 2-2**

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### **Chapter 3-1 (I)**

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